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GENETIC MANAGEMENT OF A BREEDING PROGRAMME FOR THE RIVERINE RABBIT BUNOLAGUS MONTICULARIS

MSc UP 1992

Genetic management of a breeding programme for the riverine rabbit *Bunolagus monticularis*

by

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Submitted in partial fulfilment of the requirements for the degree of

M.Sc. (Zoology)

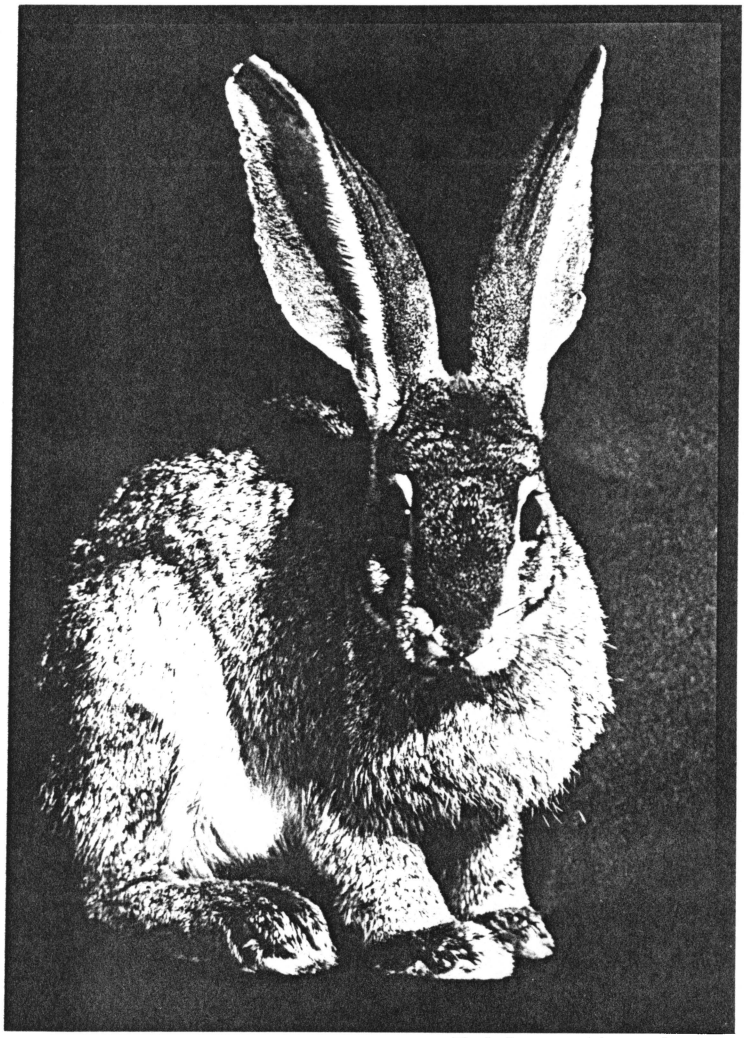
in the

Faculty of Science

University of Pretoria

Pretoria

February 1992



P.J.J. van Rensburg

"WHAT IS MAN WITHOUT THE BEASTS?

IF ALL THE BEASTS WERE GONE,

MAN WOULD DIE FROM A GREAT LONELINESS OF SPIRIT.

FOR WHATEVER HAPPENS TO THE BEAST

ALSO HAPPENS TO THE MAN.

ALL THINGS ARE CONNECTED.

WHATEVER BEFALLS THE EARTH

BEFALLS THE SONS OF THE EARTH."

(Chief Seathl. A North American Indian in a letter to the President of the United States of America - 1855).

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by

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ABSTRACT

The genetic similarity of a captive population of riverine rabbits *Bunolagus monticularis* was determined by DNA fingerprinting for assessing the suitability of individuals for inclusion in a captive breeding programme. A high degree of similarity was found among the fingerprints of individual rabbits and specimen 008 is the least inbred individual. The only male that produced spermatozoa after electroejaculation was 002 and this may indicate inbreeding depression. Although the inclusion of other individuals as founders is discussed, the initiation of a breeding programme with more than one of these captive rabbits is strongly discouraged. Fingerprint data suggest a high degree of genetic similarity of individuals within demes in the wild and the average heterozygosity of the wild rabbits is estimated at 0.02. Additional founders should thus be captured over a wide range of their geographical distribution. The genetic management of the captive breeding population is of vital importance.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to the following people and organizations:

Doctor J.W.H. Ferguson for initiating this project and obtaining the funding for it, for his continued enthusiasm and interest in my progress as well as his honesty and support;

The South African Nature Foundation for providing the funding to get the project off the ground;

Professor T.J. Robinson (Mammal Research Institute) for making his laboratory facilities available for some of the analyses and for assisting with blood culturing, as well as for a continued interest in the project;

Doctor D.J. Morris (Mammal Research Institute) for assisting in many ways throughout the project, especially with DNA fingerprinting;

Doctor H.M. Dott (Mammal Research Institute) for assisting with the collection, staining and counting of spermatozoa;

Miss A. van Dyk (De Wildt Cheetah Breeding Facility) for assisting with the capture and handling of the animals as well for her continued support for the project since my Honours degree;

Prof. D. Meltzer (Onderstepoort) for assisting with the collection of blood during the first capture

Dr. I.W. Espie (National Zoological Gardens) for assisting with the capture of animals and collection of material during other captures;

Mr. W. Labuscagne and Dr. F. Schoeman (National Zoological Gardens) for making the capture and handling of the animals possible;

Prof. P.G. Howell (Veterinary Science, Onderstepoort) for making his tissue culture facilities available;

Miss. Jenny Spencer and Mrs. G. Goosen (Veterinary Science, Onderstepoort) for assisting with the culture of tissue samples;

Andrew Duthie (Wildlife Society) for advice throughout the project;

Mr. J. Visser (Roodeplaat Research Station) for providing blood from domestic rabbits;

Mr. J. van Rensburg and B. Terblanche (GIS Laboratory) for making their laboratory facilities available and for assisting with the use of these facilities;

The University of Pretoria from whom I received a bursary;

All my friends and family, especially Karen, Marienne and Rosa, for their moral support and friendship;

My parents for their confidence in me and their continued encouragement, as well as financial support, without which I would not have been able to take the opportunity to study further;

My Creator, for giving me this opportunity and the perseverance to carry this project through.

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A: rabbits present, B: suitable habitat, rabbits absent, C: habitat destroyed, ---- 1 200m contour, ——— border between the False Upper Karoo in the east and the rest of the area where rabbits occur. 73

CHAPTER ONE

GENERAL INTRODUCTION

The riverine rabbit (*Bunolagus monticularis*) occurs in the riparian scrub along the banks of seasonal rivers of the central Karoo (Robinson 1981a,b, Duthie, Skinner & Robinson 1989). This plant community includes *Salsola glabrescens*, *Lycium* spp., *Osteospermum spinescens*, *Pteronia erythrocaetha*, *Galenia procumbens* and *Kochia pubescens* with *S. glabrescens* and *Lycium* spp. being the dominant taxa (Duthie 1989). Over the last 40 to 50 years an estimated 60% of the original habitat of riverine rabbits has been destroyed to make way for agriculture (the cultivation of wheat; Duthie 1989) and as a consequence the rabbits' range has been halved (Duthie *et al.* 1989). Estimates by Duthie (1989) suggest that the remaining habitat could possibly support 1435 rabbits but since rabbit densities vary widely along the same river the actual total population size is probably much smaller than this, indicating that the rabbits are endangered and in need of special conservation measures.

Gilpin & Soulé (1986) distinguished between deterministic and stochastic or chance extinctions. Deterministic extinctions are those that result from some inexorable change or force from which there is no hope of escape, usually occurring when something essential to the survival of a species is removed or when something lethal is introduced. Stochastic extinctions are those that result from normal, random changes or environmental perturbations. Usually, such perturbations reduce a population but do not eliminate it. Once reduced, however, the population is

at an increased risk from the same or from a different kind of random event (Gilpin & Soulé 1986). Environmental unpredictability and natural catastrophes appear to be the most critical factors limiting the persistence of natural populations of relatively large size, while demographic or genetic factors are of relatively little concern. In small natural populations, however, all of the above four factors may be important (Hedrick & Miller in press) and deterministic factors exacerbate the stochastic factors. This is the case with the riverine rabbit. The destruction of the vegetation along the banks of the Fish and Rhinoceros Rivers in the Karoo resulted in a deterministic event which may have reduced the numbers of riverine rabbits along those banks. If all the vegetation is destroyed it will probably lead to the extinction of *Bunolagus monticularis* as it is a local endemic species (Duthie *et al.* 1989). Because of the reduction in its numbers, the riverine rabbit is much more vulnerable to stochastic factors which can lead to the extinction of the species.

Any environmental change can set up positive feedback loops of biological and environmental interactions (Gilpin & Soulé 1986). These positive feedback loops may in turn have short, medium and long term effects on the population.

Short term effects of the positive feedback loops: These effects are related to the way in which population dynamics affect the survival of a population. A severe reduction in numbers makes species much more susceptible to stochastic extinctions (Gilpin & Soulé 1986). The smaller the population, the greater its vulnerability to normal, random changes or habitat perturbations

and the more frequent such perturbations the greater the probability of extinction due to fluctuations in sex ratio, population size, breeding success, mortality and other demographic parameters before the population size can recover to a safe level (Foose 1978, Gilpin & Soulé 1986).

As a result of the smaller population and the smaller distribution area, the effective population size is also reduced resulting in medium term effects coming into operation, i.e. an increase in genetic drift and inbreeding.

Medium term effects of the positive feedback loops: These effects are related to the way in which genetic characteristics affect the survival of a population. Two genetic processes undermine many aspects of individuals within a population, e.g. metabolic efficiency, growth rate, reproductive physiology and disease resistance (Crow & Kimura 1970, Ralls & Ballou 1983, Ralls, Ballou & Brownell 1983, Crow 1986, Gilpin & Soulé 1986, Soulé, Gilpin, Conway & Foose 1986, O'Brien & Evermann 1988). The first is inbreeding depression which is defined as a loss in fitness because of an increased homozygosity resulting from inbreeding (Hedrick & Miller in press) and the second, the loss of heterozygosity which results from an increase in genetic drift The degree of inbreeding depression is a linear function of the inbreeding coefficient. However, for a given inbreeding coefficient the extent of inbreeding depression is expected to be greater in a small population than a large one because of the reduced effectiveness of selection compared with genetic drift (Hedrick & Miller in press).

Three mechanisms have been offered to explain the typically reduced survivorship and fertility resulting from increased homozygosity (Packer 1979). First, increased homozygosity increases the probability of detrimental recessive alleles being expressed in a homozygous state and thus becoming phenotypically evident (the "partial dominance hypothesis", Charlesworth & Charlesworth 1987). Secondly, the heterozygote is sometimes superior to either homozygote (the "overdominance hypothesis", Charlesworth & Charlesworth 1987). This results in inbred offspring being less fit simply because they are homozygous at more loci. Thirdly, increased homozygosity decreases the variability between offspring and thus decreases the likelihood of one of them being suited to survive a sudden change in environmental conditions. It has thus been suggested that inbred offspring are at a disadvantage in nature and that behavioural patterns exist which reduce the probability of matings between close relatives (Feldman & Christiansen 1984).

Decreased viability and/or fecundity due to increased homozygosity has been found in zoo populations of the following species: Eland, *Taurotragus oryx* (Treus & Lobanov 1971), Przewalski's horse, *Equus przewalski* (Bouman 1977, Flesness 1977), Dorcas gazelle, *Gazella dorcas* (Ralls, Brugger & Glick 1980), Speke's gazelle, *Gazella spekei* (Templeton & Read 1984), the okapi, *Okapi johnstoni* (De Bois, Dhondt & Van Puijenbroeck 1990), the red panda, *Ailurus fulgens* (Roberts 1982) and the Siberian tiger, *Panthera tigris altaica* (Seal & Foose 1983).

In the case of populations not confined to zoos, apparent deleterious consequences of genetic uniformity have been

described for the cheetah (*Acinonyx jubatus*). These include great difficulty in captive breeding, a high degree of juvenile mortality in captivity and in the wild and a high frequency of spermatozoal abnormalities in ejaculates (O'Brien, Wildt, Goldman, Merril & Bush 1983, O'Brien, Roelke, Marker, Newman, Winkler, Meltzer, Colly, Evermann, Bush & Wildt 1985, O'Brien, Wildt & Bush 1986, O'Brien, Wildt, Bush, Caro, FitzGibbon, Aggundey & Leakey 1987). High frequencies of spermatozoal abnormalities due to inbreeding were also found in the lion, *Panthera leo* (Packer, Pusey, Rowley, Gilbert, Martenson & O'Brien 1991). The negative effect of inbreeding on juvenile survival has been observed in primates (Packer 1979), ungulates and small mammals (mainly rodents) (Ralls, Brugger & Ballou 1979, Ballou & Ralls 1982, Ralls & Ballou 1982a,b, Ralls & Ballou 1983), the okapi, *Okapi johnstoni* (De Bois *et al.* 1990), the gaur, *Bos gaurus* (Hintz & Foose 1982) and the European bison, *Bison bonasus* (Olech 1987).

The domestic rabbit (*Oryctolagus cuniculus*) is severely affected by artificial inbreeding and homozygosity, which result in a marked reduction in breeding performance. The percentage of animals that mate is small and the percentage of fertilized eggs is low. Litters are often small, or do not survive, and rabbits may be infertile or fail to complete pregnancy. A high frequency of malformations is also found after a number of generations of inbreeding (Chai 1979).

An increase in genetic drift and inbreeding, which reduces the heterozygosity of a population (Kimura & Crow 1963), has long term effects.

Long term effects of the positive feedback loops: These effects are associated with the inability of a population to adapt to a changed environment. Survival of a species in a changing world depends upon evolution which, in turn, is completely dependent upon the existence of a spectrum of genetic variation (Greig 1979). Genetic drift also reduces the possibility of natural selection (Gilpin & Soulé 1986). In small populations, random genetic drift can overpowers selection, making it easier to fix future mutations (Lynch & Gabriel 1990) and rendering natural selection inoperative. The loss of genetic variation reduces the probability that a population will be able to adapt to environmental changes (Gilpin & Soulé 1986). These effects result in a further reduction in population size, increased inbreeding and genetic drift, and a loss of heterozygosity which further reduces population size. The smaller the effective population size, the more rapidly genes are lost by genetic drift (Foose 1978).

Such a positive feedback system with its short, medium and long term effects can also lead to the extinction of the riverine rabbit.

Motivation:

At present, the riverine rabbit is one of South Africa's rarest and most endangered mammals (Duthie *et al.* 1989). Without the immediate implementation of a conservation programme, including a captive breeding programme, the riverine rabbit will probably become extinct as a result of the factors mentioned above.

In an attempt to prevent the extinction of these rabbits, a captive population was established at the De Wildt Cheetah Breeding Facility, with the aim of eventually releasing captive-bred rabbits back into the wild. This is considered feasible because of the presence of suitable unoccupied habitat. During 1987 five males and two females, captured on the farm Klipgat in the Karoo, were brought to De Wildt to start the captive colony. Of these rabbits, three males died in the same year. During 1988 two more males were brought from this Karoo locality but two more males died. Fortunately five kittens were born, but two of these died. Three kittens were born during 1989 and six more during 1990, bringing the population total to 16. During 1991 four rabbits died, reducing the population to 12 individuals (Van Dyk pers. comm.*). This included seven males and five females. Fourteen rabbits had thus been born and 11 had died within five years (Figure 1). The population therefore increased by only three rabbits during this period. As the entire captive population originated from four individuals (two males and two females, Figure 2), it must be significantly inbred. Domestic rabbits (*Oryctolagus cuniculus*) are particularly susceptible to the deleterious effects of inbreeding (Chai & Degenhardt 1962, Chai 1968, Chai 1969, Chai 1970, Chai 1979, Chai & Fox 1979, Chai, Kremer & Fox 1979). Although these rabbits cannot be considered very closely related to the riverine rabbit it is apparent that at least one leporid species is very sensitive to inbreeding depression. This makes it imperative to exercise special care when dealing with an endangered lagomorph such as the riverine rabbit.

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* VAN DYK, A. (personal communication). De Wildt Cheetah Breeding Facility, De Wildt.

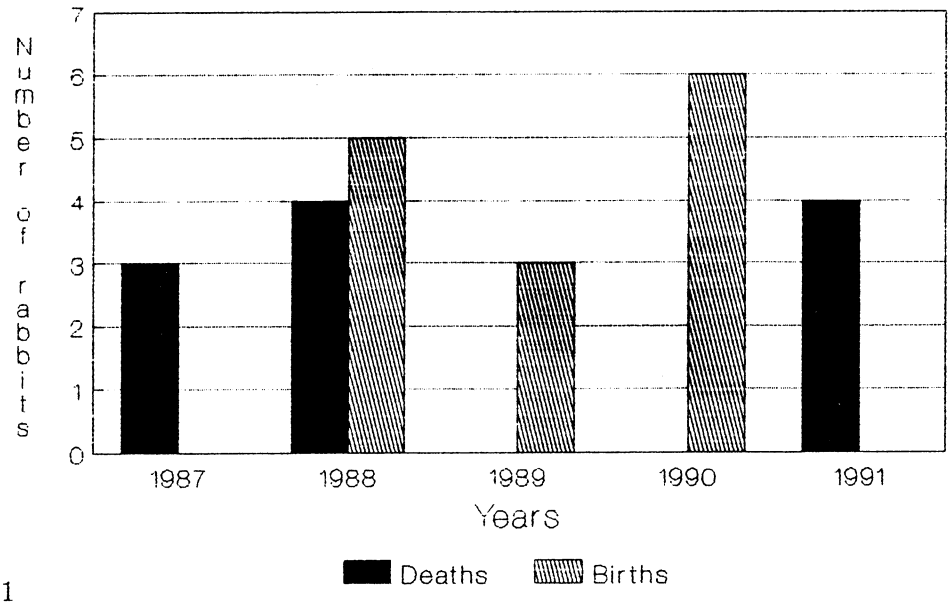

FIGURE 1

Frequency of deaths and births of the riverine rabbits at De Wildt.

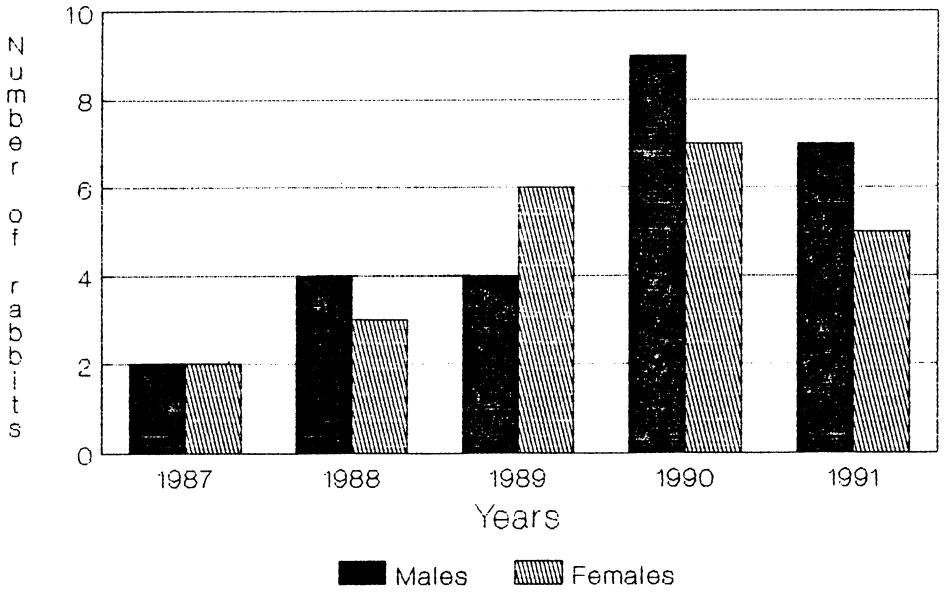

FIGURE 2

Total number of riverine rabbits at De Wildt from 1987 to 1991.

To maximize the success of the captive population, it is thus necessary to maintain maximum heterozygosity in order that the captive breeding programme achieves the minimization of inbreeding depression (Hedrick & Miller in press) thus enabling the release into the wild of animals which have the ability to resist stochastic extinctions and survive environmental change. A breeding programme for riverine rabbits in captivity has been designed (Dippenaar 1989). For the refinement and implementation of this programme it is necessary to obtain information about the genealogical relationships of the captive rabbits.

Objectives:

The aims of this study are:

1. to provide information about the genealogical relationships within the captive population of riverine rabbits. This was attempted by means of DNA fingerprinting and allowed the identification of individuals that are relatively dissimilar genetically and which would be suitable for inclusion in a breeding programme.

2. to determine the genetic similarity of the captive rabbits to that of an individual captured on the same farm during 1980. This was also performed by DNA fingerprinting and allowed speculations regarding the genetic variation within a single deme.

3. to determine if the present degree of inbreeding amongst the rabbits at De Wildt has affected the quality of the spermatozoa

in ejaculated semen.

4. to speculate about the distribution of rabbits in the Karoo, based on surveys made during 1979 and 1985. This would allow the estimation of the division of the population into subpopulations and consequently the probable heterozygosity of the population in the Karoo.

5. to make management recommendations regarding the captive breeding of the riverine rabbits at De Wildt.

CHAPTER TWO

COLLECTION OF MATERIAL AND GROWING OF CULTURES

INTRODUCTION

For obtaining DNA fingerprints it is necessary to extract DNA from the animals to be examined. DNA can readily be obtained from virtually any animal tissue. A biopsy of the liver or other body organ is very suitable for DNA extraction. However, because riverine rabbits are endangered it was not possible to sacrifice specimens and DNA could only be obtained by relatively non-invasive sampling from live rabbits. The only available material for DNA extractions was thus from blood and ear clippings. Blood was drawn from all individuals for this purpose. For some rabbits the amount of DNA extracted was insufficient for DNA fingerprinting due to blood coagulation or insufficient blood taken. More DNA was therefore necessary and this was obtained through culturing of white blood cells or ear clippings.

Blood and tissue cultures:

Animal cells in culture display the same type of growth pattern as micro-organisms. In particular, they show the classical growth kinetics demonstrated by cultures of bacteria, yeasts and protozoa. When cells are taken from a stationary culture there is at first a lag phase of some hours to some days before growth commences. Growth then proceeds steadily with the population doubling every 15 to 20 hours in the case of fast growing cells. This is known as the logarithmic phase. The maximum population is

then reached and the cells enter a stationary phase (Paul 1975).

The principle of lymphocyte culture is based on the incubation of whole blood, leukocytes, or lymphocytes alone in a suitable medium. The culture of whole blood utilizes all available lymphocytes, which usually constitute 25 - 50% of the leukocyte count (Pfeiffer 1974).

Mitogens:

Mitogens can be added to the culture medium to stimulate cell division. Two types of mitogens were tested.

PHA: Phytohemagglutinin (PHA), a plant-derived mucoprotein is considered to be the most reliable mitogen for human and animal lymphocytes. It activates immunocompetent lymphocytes and elicits cell division. The mitotic rate can amount to 1% per hour at 72 hours. However, there seems to be a threshold value for the efficacy of PHA. The number of activated cells increases with the PHA concentration until all immunocompetent lymphocytes are activated (Pfeiffer 1974).

IL 2: Interleukin 2, a hormone-like growth factor, is secreted after antigenic or mitogenic stimulation of T lymphocytes (Smith & Ruscetti 1981). Consequently it can be used to stimulate the growth of leukocytes in a culture instead of PHA or in conjunction with PHA.

Mitosis is observable in the leukocyte culture after two days, although the mitotic rate only achieves optimal values after 2.5

- 3 days at constant temperature. Variations in the mitotic rate depend on the type of PHA used and on the temperature (Pfeiffer 1974).

MATERIALS AND METHODS

Capture and sample collection:

A: Domestic Rabbits

Blood Collection: In order to become acquainted with the protocols associated with DNA extraction and DNA fingerprinting, these techniques were first attempted using blood of domestic rabbits. Blood was drawn, once at the Zoology Department, twice at Roodeplaat Research Institute and once at Medunsa, from inbred domestic rabbits with unknown genealogical relationships (Visser pers. comm.*). Blood was collected by ear bleeding into acid-citrate dextrose (ACD) tubes. This was found to be the most effective anti-coagulant and has previously been used for the collection of rabbit blood (e.g. Bellen, Van de Weghe, Bouquet & Van Zutphen 1984). Eight to 12ml of blood was collected from each rabbit without immobilising the animals.

The blood samples were immediately centrifuged for 10 min. at 3000 rpm. The buffy coats were collected and immediately frozen in liquid nitrogen. They were then stored at -70^{o}C until DNA extractions were performed.

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* VISSER, J. (personal communication). Roodeplaat Research Station.

Blood Cultures: Blood was also drawn by ear bleeding under sterile conditions into heparinised tubes (e.g. Andersson, Bengtsson, Hellman, Kallman & Ranje 1985) from domestic rabbits at Onderstepoort. From these samples, it was attempted to grow blood cultures in order to obtain sufficient leukocytes from which enough DNA could be extracted to perform fingerprinting.

B: Riverine Rabbits

Capture: Fifteen riverine rabbits were captured at De Wildt on January 4th 1991. Each rabbit was marked by tattooing three digits on the inside of the left ear using Hauptner Special tattooing ink. The numbers ranged from 001 to 015.

Rabbits were anaesthetized with Halothane in order to collect samples without causing excessive stress. After sampling the rabbits were kept in holding boxes to ensure complete recovery from anaesthesia before release into the holding camp.

On June 6th 1991, eleven of the rabbits were recaptured, as was an extra rabbit not captured on the first occasion and which had not been marked by means of tattooing. Of the four rabbits not recaptured, two had died (identity unknown since tattoe marks were not checked at death) and two were not sighted again. All the rabbits were marked again according to the standard ear clipping numbering system of the Zoological Gardens (Figure 3) (Espie pers. comm.*).

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* ESPIE, I.W. (personal communication). National Zoological Gardens, Pretoria.

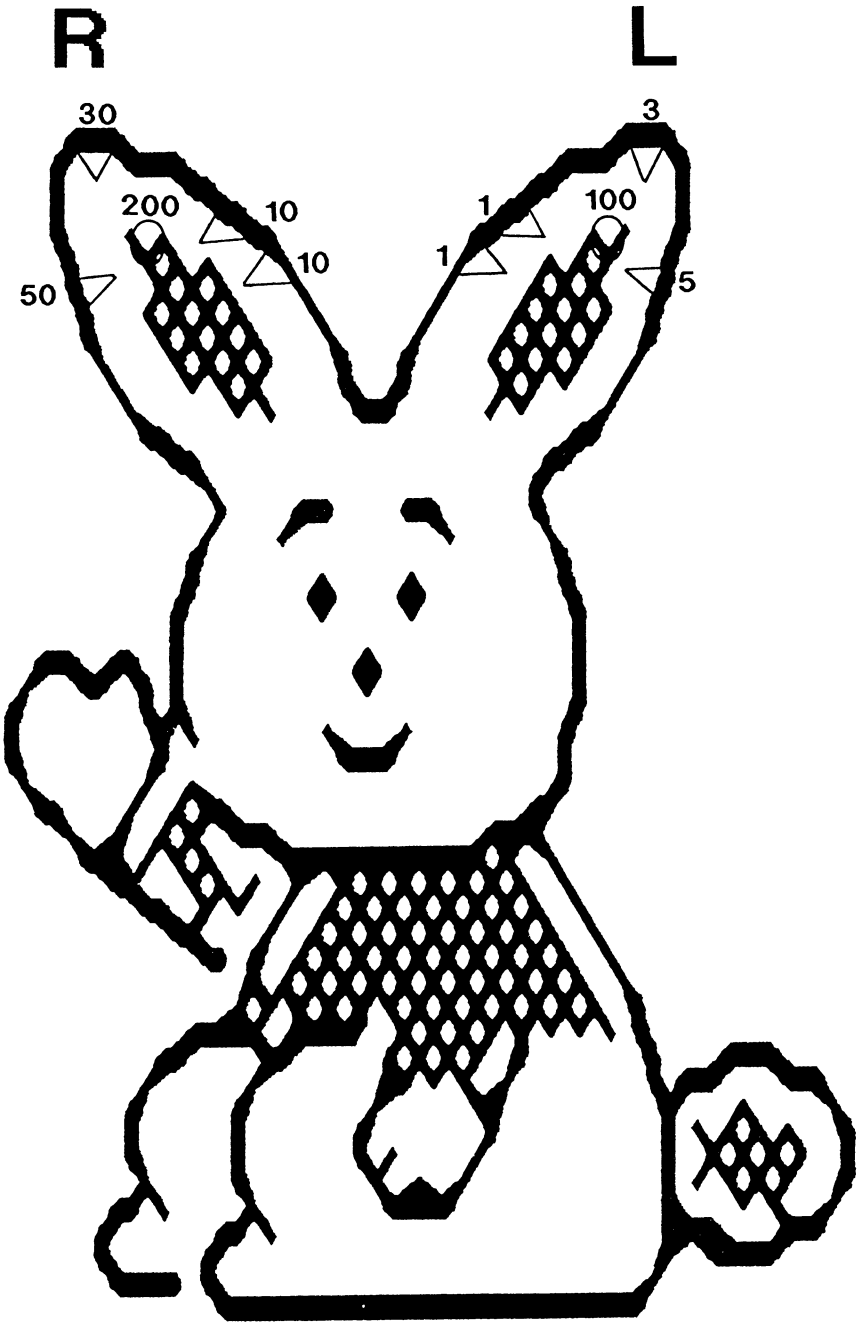

FIGURE 3

The standard ear clipping numbering system of the Zoological Gardens.

On November 7th 1991, the twelve rabbits caught in June were recaptured. Electronic subcutaneous transponders (Trovan) were implanted on the left shoulder blade of all the rabbits. The individual identification marks of the captive rabbits after this third capture are indicated in Table 1

TABLE 1

The individual identification and gender of riverine rabbits in captivity at De Wildt Cheetah Breeding Facility up till January 1992.

Tattoo Nr	Ear Clipping Nr	Electronic Nr	Sex
001	38	00-001D-7F8E	F
002	130	00-001C-4757	M
003	Missing	Missing	M
004	13	00-001C-B8FC	F
005	130	00-0020-D7EB	F
006	40	00-0020-24F6	M
007	83	00-0021-57D9	F
008	83	00-0021-35E0	M
009	Missing	Missing	F
010	Missing	Missing	M
011	Missing	Missing	F
012	140	00-001C-BD8F	M
013	36	00-001D-8687	M
014	85	00-0021-2B0B	M
015	38	00-0020-C39B	M
Missing(016)	33	00-001C-BDCF	F

Rabbit 012 was probably one of the original males, brought to De Wildt during 1987, since it had been marked with a hole in the middle of the ear.

Blood Collection: Blood was collected in the same way as for domestic rabbits.

A 22 gauge needle was used during the first capture in January 1991 and the blood dripped straight into an ACD tube. The blood and ACD were continuously mixed. Three to five ml of blood was collected from every individual and treated as for the domestic rabbits.

Since sufficient DNA could not be extracted from the buffy coats, the rabbits were recaptured in June 1991 and additional samples collected. Blood was collected by ear bleeding, but this time by means of butterfly needles. Since rapid blood coagulation caused severe problems for DNA extraction, the blood was drawn into a syringe containing the ACD and continuously mixed as thoroughly as possible with the anti-coagulant. A minimum of 5ml of blood was drawn from every individual. The whole blood was transported back to the laboratory on ice. DNA extractions were performed immediately for eight of the twelve samples. The other four samples were left at -20^{o}C until DNA extraction five days later.

Tissue Collection: Two ear clippings were collected from each riverine rabbit during the first capture and immediately frozen in liquid nitrogen. These were stored at -70^{o}C. During the second capture two ear clippings of each rabbit were again collected. The size of these clippings was approximately 25mm^{2}.

The ear was first sterilized by washing it with 70% EtOH before clipping. The edge of each clipping was cut off to remove the long hair and prevent contamination in culture. The ear clippings were placed in tissue culture medium consisting of MEM, 5% FCS (Fetal calf serum), 0.24mg/ml penicillin, 0.4mg/ml streptomycin and 0.005mg/ml fungizone. In the laboratory, the ear clippings were transferred to storage medium which consisted of MEM, 15% FCS, 0.1mg/ml penicillin and 0.2mg/ml streptomycin. The first batch of clippings were minced and cultured four days later. A second batch was cultured eight days later.

Blood and Tissue Cultures:

Blood Cultures: In an attempt to grow sufficient white blood cells for obtaining a reasonable amount of DNA, blood cultures of domestic rabbits were attempted. Blood was drawn from the rabbits under sterile conditions into heparinised tubes.

Two culture mediums were tested:

1. **HAM's F10**: Ham's medium was originally designed especially for cloning cells. However the low concentrations of some amino acids limit the yields (Paul 1975). All these attempts were performed with Fetal Calf Serum. Table 2 illustrates the variable conditions for the nine different samples.

2. **RPMI**: The second medium was developed for leukocyte culture (Paul 1975). Each tube contained 1.25 ml serum (either FCS or autologous serum), 5 ml stock medium and 0.5 ml buffy coat. The stock medium consisted of 2.5ml IL 2, 0.25ml PHA, 0.15 Na Heparin and 50ml RPMI. The buffy coat (BC) of each of four samples was

halved before adding it to the test tubes. Consequently the eight culture tubes contained stock medium as well as buffy coat and either of the two different sera (Table 3).

The cultures were incubated for 72 hours at 37°C. Afterwards the supernatant was removed and the pellet washed in 0.9% sterile saline solution and stored at -70°C until DNA extraction.

Samples kept under those four conditions believed to be the most successful were used for DNA extractions. These included PHA with Buffy Coat (BC) and IL 2 with BC in HAM's F10 (Table 2) as well as BC with FCS and BC with autologous serum in RPMI (Table 3).

Tissue Cultures: A procedure similar to that used by Robinson (1981c) was used to culture ear biopsies of the riverine rabbits. Samples from a wild riverine rabbit were taken in 1980, and fibroblast cells grown by T.J. Robinson were subsequently stored in liquid nitrogen. Growth of fibroblasts from this culture was restarted in MEM and 15% FCS.

After three days one ear clipping of every individual was minced under sterile conditions and placed into small tissue flasks. Culture medium (6 ml MEM, 15% FCS & 1ml/lGentamycin) was added and the sealed flasks incubated at 37°C.

After seven days the second ear clipping of every individual was minced and placed into small tissue culture flasks with 2.5 ml MEM, 1ml/l Gentamycin, 1ml/l Fungizone and 15% FCS and incubated at 37°C.

TABLE 2

The different conditions for the nine samples with the first medium (HAM's F10).

Samples	Blood/BC	Amount of	Mitogen
1	Blood	15 drops	PHA
2	Blood	0.25ml	PHA
3	BC	0.5ml	PHA
4	Blood	15 drops	IL 2
5	Blood	0.25ml	IL 2
6	BC	0.5m	IL 2
7	Blood	15 drops	PHA & IL 2
8	Blood	0.25ml	PHA & IL 2
9	BC	0.5ml	PHA & IL 2

PHA - Phytohemagglutinin

IL 2 - Interleukin 2

FCS - Fetal Calf Serum

BC - Buffy Coat

TABLE 3

The contents of the different test tubes with the second medium (RPMI).

A:	first half BC & FCS	B:	second half BC & autologous serum
C:	first half BC & FCS	D:	second half BC & autologous serum
E:	second half BC & FCS	F:	first half BC & autologous serum
G:	second half BC & FCS	H:	first half BC & autologous serum

After the cells began growing, small clusters of cells were visible at the bottom of the flasks. In order to promote growth, these were separated and spread in new flasks by means of trypsinization (Rous & Jones 1916). Trypsinization was performed after the medium had been poured out and the cells washed with phosphate-buffered saline (PBS^-). Active Trypsin Versine (ATV, 2ml) with 8ml sterile distilled water was then added and the flasks were re-incubated at 37°C for 2 minutes. A small amount of medium was added to transport the disaggregated cells to the new flask. More medium was added to the new flask and left in the incubator.

The medium of all the flasks was changed at least once a week to ensure that the pH would remain within the range with sufficient nutrients for cell growth. After three weeks the six cultures that survived were placed in McCoy/L15, 1ml/l Gentamycin and 15% FCS because this is a more nutritious medium than MEM. Cells were harvested with PBS^- after tripsinization and washed with 0.9% sterile saline solution. The pellet of fibroblasts was left at -20°C until the DNA extraction was performed.

RESULTS

Blood Cultures:

DNA extractions of domestic rabbits were performed on two of the samples of the first experiment (Table 2), PHA with BC and IL II with BC, and two of the second experiment (Table 3), BC with FCS and BC with autologous serum. Only the samples with the BC were chosen because it was believed that these samples were most

likely to have grown enough white blood cells to yield a large enough amount of DNA for fingerprinting.

Unobservable amounts of DNA were obtained from the blood cultures. Although this might have been high molecular weight DNA, it was insufficient for fingerprinting.

Tissue Cultures:

Four T75 flasks of confluent fibroblasts of the 1980 rabbit were harvested and stored at -20°C until DNA extraction was performed. Four T75 flasks of confluent fibroblasts of rabbit number 012 were also harvested after seven weeks and stored at -20°C until DNA extraction. These cultures yielded a relatively large amount of high quality DNA. Unfortunately, all the other cultures died, leaving blood as the only source of DNA for the other individuals.

DISCUSSION

Blood Cultures:

Since the addition of heparin to PHA containing medium does not prevent the agglutination of erythrocytes in individual compact and solid clots, it must be expected that lymphocytes within the clots will be under poor culture conditions. In order to avoid agglutination the cultures have to be shaken twice a day (Pfeiffer 1974). The large number of erythrocytes may also have a detrimental effect, especially during fixation, because the erytrocytes very easily form clumps which must then be dispersed

with the pipette (Pfeiffer 1974).

The fact that most of the collected blood in the heparin tubes started to coagulate while being transported to the laboratory might be the main reason for the very small amount of DNA extracted. This is because large numbers of leukocytes could have been lost by being included in coagulated blood. It might also be that there are simply not enough leukocytes after culturing to yield sufficient DNA for fingerprinting after extraction.

Tissue Cultures:

The reasons for the failure to grow the ear clippings may be due to inexperience. With the first batch of ear clippings, conditions may not have been sufficiently aseptic. For instance, the ear clippings were not sterilized a second time with 70% EtOH before mincing. This might be the reason for the large number of fungal or bacterial contaminations that occurred. These contaminated cultures were discarded, without attempting to sterilize them by treatment with antibiotics and fungizone.

Cultures may have started growing and then died after a week or two because the medium was not changed often enough. The pH would thus not have been prevented from exceeding the lower limit of the optimum and this would have deleterious effects on cell growth. Another factor that might have contributed to cell death after initial growth was the length of time that elapsed during trypsinization. Long exposure to trypsin damages the cells and might result in the cells dying off.

Although the tissue cultures yielded a relatively large amount of high quality DNA, it was impossible to grow more cultures. More samples could not be obtained due to the problems of stressing the rabbits during handling and due to difficulties in organizing a capture operation. Fortunately, tissue cultures were obtained for the individual from which the blood sample had yielded low quality DNA as well as for the 1980 individual for which no blood sample existed.

CHAPTER THREE

DNA FINGERPRINTING

INTRODUCTION

DNA fingerprinting has been used for different purposes: forensic studies (Gill, Jeffreys & Werrett 1985), segregation analysis and linkage studies (Burke & Bruford 1987, Georges, Lathrop, Hilbert, Marcotte, Schwers, Swillens, Vassart & Hanset 1990, Brock & White 1991), parentage assessment (Burke & Bruford 1987, Wetton, Carter, Parkin & Waltrs 1987, Burke, Davies, Bruford & Hatchwell 1989, Birkhead, Burke, Zann, Hunter & Krupa 1990, Ely & Ferrell 1990, Inoue, Takenaka, Tanaka, Kominami & Takenaka 1990, Westneat 1990, Hoelzel, Ford & Dover 1991, Jones & Mench 1991, Packer, Gilbert, Pusey & O'Brien 1991, Tegelström, Searle, Brookfield & Mercer 1991, Wolfes, Måthé & Seitz 1991), determination of relationship (Jeffreys, Brookefield & Semeonoff 1985, Hill 1986, Amos & Dover 1991, Packer, Gilbert *et al.* 1991), determination of genomic diversity and population history (Yuhki & O'Brien 1990) and the determination of genetic variation within and between populations (Faulkes, Abbott & Mellor 1990, Gilbert, Lehman, O'Brien & Wayne 1990, Hoelzel & Dover 1991, Packer, Pusey *et al.* 1991, Reeve, Westneat, Noon, Sherman & Aquadro 1990). In this study DNA fingerprints were used in order to determine the amount of genetic variation within the population of captive riverine rabbits at De Wildt and to attempt to identify specific rabbits that would be suitable for inclusion in a captive breeding programme. Specimen selection was done on the basis of genetic dissimilarity, as determined from fingerprint data.

A significant proportion of the genome of most higher eukaryotes consists of micro/minisatellites (Kashi, Tikochinsky, Genislav, Iraqi, Nave, Beckmann, Gruenbaum & Soller 1990). These are thought to be non-coding DNA sequences comprising multiple copies of a sequence of typically less than 65 base pairs in length and are dispersed throughout the genome (Burke & Bruford 1987, Jeffreys, Wilson, Kelly, Taylor & Bulfield 1987, Georges, Lequarré, Castelli, Hanset & Vassart 1988, Burke 1989). Although it is not yet known how these DNA fragments arose they are not derived from a single localized region of one autosome (Jeffreys, Brookefield & Semeonoff 1985).

Minisatellite loci are considered hypervariable because they include the most polymorphic sequences ever detected. Polymorphism results from allelic differences in the number of repeats (Jeffreys, Wilson & Thein 1985a, Devor, Ivanovich, Hickok & Todd 1988). Due to this tremendous variation in the number of repeats within a tandem sequence when comparing a specific site between different individual organisms, the sites have been termed Variable Number of Tandem Repeats (VNTRs) loci (Nakamura, Leppert, O'Connell, Wolff, Holm, Culver, Martin, Fujimoto, Hoff, Kumlin & White 1987). This hypervariability is the result of a high mutation rate resulting in loss or gain of repeats at a particular site. It appears unlikely that meiotic or mitotic crossing-over between alleles is a major force in the generation of new mutants (Pemberton & Amos 1990). Within-allele processes, such as unequal crossing-over between sister chromatids, are favoured by Jeffreys, Neumann & Wilson (1990) as the mechanism responsible for generating changes in repeat-unit number. The mutation rate increases with heterozygosity and the relationship

between mutation rate and heterozygosity is in accordance with the theoretical predictions of the neutral mutation/random drift hypothesis (Jeffreys, Royle, Wilson & Wong 1988). The mean mutation rate to new length alleles in DNA fingerprints detected by multi-locus polycore probes has been estimated at about 0.002 to 0.05 per DNA fragment per gamete in captive chimpanzee, human, cattle and eusocial naked mole-rat DNA (Jeffreys *et al.* 1988, Georges *et al.* 1990, Reeve *et al.* 1990, Ely, Alford & Ferrell 1991). The polymorphism in the VNTRs is the key to the development of DNA identity patterns (Dodd 1985).

Jeffreys *et al.* (1985a) and Jeffreys (1987) discovered a family of minisatellite sequences comprising of tandem repeats of a 33 base pair unit, including a core sequence that consists of an almost invariant sequence GGGCAGGAXG preceded by a 5bp sequence common to most, but not all, repeats. These repeat sequences can be used to design probes for detecting individual polymorphic minisatellite regions. In fact, the shared core sequence, which possibly serves as a recombination signal, can be used for the simultaneous analysis of many hypervariable sites (Jeffreys *et al.* 1985a).

The degree of genetic relatedness (Table 4), as reflected by the proportion of shared fragments within a group of related individuals (pedigree), is important for wildlife population studies. Markers in the heterozygous state can be used to trace genes from parents to offspring. The reverse procedure may also be considered: genotypic markers in offspring must have been inherited from the parents unless a germ cell mutation occurred (Burke & Bruford 1987, Schäfer, Zischler, Birsner, Becker &

Epplen 1988, Kirby 1990, Amos & Dover 1991). It is thus possible to infer the parentage of an individual.

TABLE 4

The degree of genetic relationship between pedigree members and the proportion of shared genes (Kirby 1990). The coefficient of inbreeding is usually defined as the probability of identity by descent (Ballou 1983, Crow 1986). The proportion of genes in common suggests the expected number of shared ministellite sites when considering DNA fingerprints of the individuals in question. This is expected in ideal circumstances without inbreeding.

Genetic Relationships

Relationship between individuals	Degree of relationship	Proportion of genes in common	Coefficient of inbreeding(F)
Monozygotic twins	Identical	1	
Dizygotic twins	First	1/2	1/4
Siblings	First	1/2	1/4
Parent-child	First	1/2	1/4
Aunt/Uncle-niece/nephew	Second	1/4	1/8
Half siblings	Second	1/4	1/8
Double first cousins	Second	1/4	1/8
First cousins	Third	1/8	1/16
Half-uncle niece	Third	1/8	1/16
First cousins once removed	Fourth	1/16	1/32
Second cousins	Fifth	1/32	1/64

DNA from any somatic tissue in the body is suitable for identity testing because DNA is identical in all cells except those with mutations (Jeffreys, Wilson & Thein 1985b, Kirby 1990, Buitkamp, Ammer & Geldermann 1991). DNA fingerprints indicate a clear pattern of Mendelian inheritance for VNTRs (Dodd 1985, Wong, Wilson, Jeffreys & Thein 1986, Kashi *et al.* 1990) and are more

precise for relationship studies than classic protein polymorphisms (Lewin 1989). The estimated fraction of an eukaryotic genome needed to code for all the amino acid sequences in an organism is about 0.5% and excludes introns and leader sequences (Powell 1983). Since DNA fingerprinting, as opposed to the measurement of protein polymorphism, detects variation in the non-coding DNA, this technique allows the detection of more genetic variation. (Watson, Hopkins, Roberts, Steitz & Weiner 1987).

The procedure of DNA fingerprinting is as follows: An individual's DNA is extracted from a tissue sample (Miller, Dykes & Polesky 1988) and cleaved with a restriction endonuclease which cuts the DNA at recognition sites containing a specific short sequence of nucleotides to produce fragments differing in length (Burke 1989). These variants in restriction fragments produced after restriction digestion of DNA are called restriction fragment length polymorphisms (RFLPs). The resulting fragments are separated according to molecular weight by gel electrophoresis (Devor *et al.* 1988) and each will appear as a different-sized band on an autoradiogram of an individual's DNA profile (Jeffreys, Brookefield & Semeonoff 1985, Kirby 1990, Buitkamp, Ammer *et al.* 1991). Once separated, the double-stranded DNA fragments are denatured into their component single strands and permanently transferred to a filter membrane by Southern blotting (Southern 1975). Nylon filter membranes exibit increased sensitivity and are more resilient, easier to handle, can withstand multiple hybridizations without fragmentation and are thus more advantageous than nitrocellulose membranes (Twomey & Krawetz 1990).

Specific regions of the DNA are then detected and characterized by using a previously cloned DNA sequence as a probe for detecting a particular family of VNTRs (Burke 1989, Kirby 1990). The probe DNA is radioactively labeled and denatured (made single-stranded). In solution it binds (hybridizes) to those restriction fragments on the membrane that contain complementary VNTR sequences. The amount of genomic DNA needed to generate a detectable hybridization signal depends on a number of factors, including the proportion of the genome that is complementary to the probe, the size of the probe and its specific radio activity, and the amount of genomic DNA transferred to the filter. The restriction fragments are finally revealed as bands on an autoradiograph by exposing X-ray film to the hybridized filter (Burke 1989), or as visible blue bands by making use of a biotinilated probe. The latter procedure has the advantages of being safer and quicker, and the labeled probe can be stored for a long time and be reused many times (Medeiros, Macedo & Pena 1988).

There is considerable heterogeneity in the extent of interindividual variation, depending on the particular probe-species combination. Consequently, it seems difficult to compare the results obtained in different species and to use them to draw conclusions on, for instance, inbreeding or mutation rate (Georges *et al.* 1988). An assumption often made in the application of DNA fingerprints is that the matching of DNA fragments identified with one probe or at one genetic locus is independent of matching fragments identified with any other probe or at any other genetically unlinked locus. This may not be valid (Cohen 1990).

If a multilocus DNA analysis approach is used, a match of two sets of DNA fingerprints normally implies that they are from the same individual because each person's VNTR organization is unique. If the fingerprints derive from the same individual the probability of a match is close to one; if they derive from different individuals, the probability of a match (from experience to date) is close to zero. Thus only a few fragments are shared between two randomly selected individuals. The probability of shared bands increases for smaller VNTRs, probably resulting from lower genetic variability (higher allele frequencies) of these loci combined with the fortuitous co-migration of similar-sized minisatellite fragments (Jeffreys *et al.* 1985b). Population studies can be carried out to estimate the probability that any specific fragment in a multiband profile present in individual A is also present in another individual B randomly selected from the population. This mean probability of band sharing varies, and was determined as 0.19 - 0.26 for Northern European people, Japanese people and birds (Jeffreys, Brookfield & Semeonoff 1985, Burke & Bruford 1987, Honma & Ishiyama 1989). The ability of DNA fingerprinting to statistically identify parents or to establish degrees of relatedness between individuals depends on this probability (Honma & Ishiyama 1990, Keane, Waser, Danzl-Tauer & Minchella 1991). Provided the loci analyzed are not linked, the probability that all fragments present in individual A are also present in individual B can be calculated (Kirby 1990). If linkage exists the number of bands that can provide useful information for the resolution of paternity or kinship is fewer than the observed number of bands (Buitkamp, Zischler, Epplen & Geldermann 1991, Hoelzel *et al.* 1991). The DNA fingerprints can be used to measure

the probability of paternity which is based upon statistical frequency of shared bands and the paternity index (Dykes 1988, Kirby 1990). However, DNA fingerprints are phenotypes, not genotypes, and the precise aetiology of different bands is usually not known (Hedrick & Miller in press).

Difficulties:

Although DNA fingerprinting is suitable for determination of relatedness among first order relatives, it does not provide a powerful means of assessing individual relationships beyond (and often including) second-degree relationships (Lynch 1988). Three technical difficulties in using DNA fingerprinting to obtain individual estimates of relatedness have been identified:

(1) the upward bias of fingerprint similarity compared with relatedness caused by finite numbers of alleles,

(2) the inability to completely correct for such bias because of its individual specificity, and

(3) the sampling variance caused by variation in identity by descent within and between loci.

Additional problems are co-migration of non-allelic markers, linkage and/or linkage disequilibrium between marker loci, the frequent inability to observe markers with very low molecular weights, possible linkage of marker loci with other loci under selection, and high and variable mutation rates (Jeffreys *et. al.* 1988). If these are taken into account, it is clear that considerable caution needs to be exercised in applications of DNA fingerprinting to estimate individual relatedness (Lynch 1988).

With very large numbers of alleles per locus (the critical number

increasing with the distance of the relationship), the bias of DNA fingerprint similarity compared with relatedness can be ignored. However, while most VNTR loci do appear to be exceptionally variable, the number of alleles per locus is by no means high enough to permit the use of similarity as a reasonable estimator of relatedness (Lynch 1988). It is also possible that only a few loci are visible as fragments on the fingerprint since the majority of the resolved bands might be allelic and many might be cosegregated as single-locus Mendelian alleles through successive generations (Brock & White 1991).

In some applications, such as the identification of optimal breeding pairs in genetic conservation programmes, it will often be less critical to establish absolute relatedness than rank-order relatedness. Since expected similarity is a monotonic function of genealogical relatedness, DNA fingerprinting can provide a useful guide to such decision making (Lynch 1988).

DNA fingerprinting is very expensive and research objectives must be clearly specified before starting (Weatherhead & Montgomerie 1991).

MATERIALS AND METHODS

DNA extraction:

High molecular weight genomic DNA was isolated from whole blood and cultured fibroblasts. DNA extractions were performed according to the salting out method (Miller *et al.* 1988) with some modifications. Whole blood was first washed with a sucrose-

triton-X lysis buffer (Sucrose/Tris/$MgCl_2$/Triton X-100) to eliminate the red blood cells. Then the cell lysates were digested overnight at 42°C - 50°C with $T_{20}E_5$ (Tris/EDTA), 10% SDS and a proteinase K solution (Prot K/SDS/EDTA). The next morning saturated NaCl was added to precipitate the protein pellet. The DNA was precipitated by adding absolute ethanol to the supernatant. The DNA precipitate was removed with a pasteur pipette and transferred to a microcentrifuge tube. If the DNA could not be removed with a pipette, it was left at -20°C for at least four hours in order to precipitate and then centrifuged. The DNA was rinsed in cold 70% ethanol before dissolving it in $T_{10}E_1$ (Tris/EDTA) for one day at room temperature.

The same procedure was followed for extractions using fibroblasts except for the washes used to eliminate red blood cells. The procedure started at the overnight digestion with $T_{20}E_5$, 10% SDS and the proteinase K solution.

Determination of DNA concentration and purity:

After DNA extraction the DNA concentration and purity was determined using both a spectrophotometer and trial agarose gel electrophoresis in the presence of ethidium bromide. With the spectrophotometer the DNA concentration of each sample was measured by measuring the optical densities at wavelengths of 260nm and 280nm. The reading at 260nm was used to calculate the concentration, while the OD_{260}/OD_{280} ratio was used as an estimation of the purity of the DNA. A pure preparation of DNA has an OD_{260}/OD_{280} of 1.8. If there is contamination with protein or phenol, the ratio will be significantly less and an accurate

quantitation of the amount of nucleic acid will not be possible (Maniatis, Fritsh & Sambrook 1982).

The trial electrophoresis was performed using 100ml agarose (0.7%) and 3ul of ethidium bromide (10mg/ml). A continuous 1x TBE (Tris-base/Boric acid/EDTA) buffer was used. Bromophenol Blue (2ul) was added to each DNA sample (3ul) before loading in order to monitor the migration of the DNA. The trial gels were used to determine the amount of high molecular weight DNA. Undigested DNA is run on the trial gel and thus the DNA is expected to show a distinct clear band at the top of the gel when placed on a transilluminator (Spectroline - 312nm Ultraviolet). RNA and degraded DNA usually run in front of the Bromophenol Blue dye. Following these procedures the DNA was diluted in additional $T_{10}E_1$ to a final volume of 1ug/ul.

Restriction endonuclease digestion of DNA:

DNA was digested with the restriction enzyme HaeIII (Boehringer Mannheim Biochemicals) following the protocol outlined by the manufacturer. This enzyme and HinfI (Boehringer Mannheim Biochemicals) were selected according to their documented suitability (e.g. Faulkes, Abbott & Mellor 1990, Buitkamp, Ammer *et al.* 1991, Buitkamp, Zischler *et al.* 1991, Hoelzel & Dover 1991, Keane *et al.* 1991, Tegelström *et al.* 1991, Wolfes *et al.* 1991). Because of the very small amount of DNA extracted from some individuals and because better results were obtained with HaeIII than with HinfI on domestic rabbits, the former enzyme was chosen for the single and final digest, of riverine rabbit DNA.

Five to ten ug of a 1ug/ul DNA solution was used for the digestion. A compliant buffer to provide the cofactors and to correct the pH, as well as spermidine free base (0.1M trihydrocloride) which aids with the digestion of the DNA, were added to the solution. Three to five units of enzyme per ug of DNA was added. Sterile distilled water was used to make up the solution to a final volume of 35ul.

The solution was incubated at 37^{o}C overnight. The following morning 1ul enzyme was added to ensure complete digestion and the solution was left at 37^{o}C for a further 2 - 3 hours. Thereafter 1/10 Bromophenol Blue solution, a high density loading dye, (Bromophenol Blue/EDTA/glycerol) was added and the samples were left at -20^{o}C until loading onto the gel.

Agarose gel electrophoresis of DNA fragments:

Fragments were separated in 1% standard-sized agarose gels at a constant voltage of 40V in 1x TBE buffer for 40 hours. Ethidium bromide was added to the agarose, before setting, to visualize the digested DNA on a transilluminator. A mixture of two DNA molecular weight markers were used to size the hybridizing DNA fragments: bacteriophage lambda digested with HindIII as well as HindIII + EcoRI (Thein & Wallace 1986). The fragments of the mixed markers spanned the range 4 to 23 kilobases. The buffer was changed after 24 hours to prevent the effect of heating and after running, the gel was photographed on the transilluminator with 50 ASA film.

Southern Blotting:

Gels were denatured (NaCl/NaOH), rinsed with distilled water and neutralized (NaCl/Tris-base/EDTA, pH 7.2) twice. Fragments were transferred to Hybond-N nylon membranes (Amersham) by Southern blotting (Southern 1975). The blotting protocols for Hybond membranes, as prescribed by the manufacturer, were followed. The filters were subsequently stabilized in SSC and baked at 80°C for two hours to permanently bind the DNA to the membrane.

The membranes were probed with two independent probes: a non-isotopic alkaline phosphatase (AP) flanking probe purchased as a commercial kit (SNAP, Molecular Biosystems, Inc.) and the myoglobin flanking consensus core sequence (pUCJ). The latter consists of approximately 25 tandem copies of the following repeat sequence (5') GGAGGTGGGCAGGAAG (3') (Georges *et al.* 1988).

SNAP hybridization:

Membranes were first hybridized with the SNAP probe, following the protocol outlined by the manufacturer. The wash buffers (SSC/Triton X-100, pH 7.0 and SSC/SDS, pH 7.0) were preheated to 50°C. The membrane was prehybridized in hybridization buffer (SSC/BSA (Bovine Serum Albumin)/SDS, pH 7.0) at 50°C for 10 minutes. The hybridization solution was prepared by adding SNAP probe to fresh hybridization buffer. The hybridization buffer used in the prehybridization step was decanted and the premixed hybridization solution added. The membrane was incubated at 50°C for 15 minutes. Thereafter the membrane was removed from the bag in which it was hybridized and washed twice in prewarmed SSC/SDS

at 50°C for 5 minutes. This was followed by two successive washes in prewarmed SSC/Triton X-100 at 50°C for 5 min. each. Subsequently two successive washes in SSC were performed at room temperature for 5 minutes. Following this the membrane was ready for colour development. The colour substrate buffer (NBT/BCIP/Alkaline Phosphatase Buffer) was prepared shortly before use and kept in the dark as much as possible because of its light sensitivity. It was added to the membrane in a clean bag, which was then sealed, wrapped in aluminium foil and put into a dark coloured envelope to protect the bag from exposure to light. The membrane was incubated overnight at 37°C. Following complete colour development the membrane was washed with tap water for at least 3 min. and allowed to air dry. This resulted in purple bands on the membrane which were then photographed.

Following hybridization with SNAP DNA, the membranes were stripped by denaturation, rinsing in distilled water and neutralization. This was repeated before stabilization of the membranes in 2x SSC. This allowed the same filters to be reprobed using the ^{32}P dCTP labeled pUCJ.

Preparation of pUCJ probe:

The probe was supplied as a small amount of purified plasmid DNA.

Transformation: Dr D.J. Morris used *Escherichia coli* strain HB101 (a hybrid strain between *E. coli* K-12 and *E. coli*; Boyer & Roulland-Dussoix 1969) to transfer the plasmid by transformation (Maniatis *et al.* 1982). Bacterial cell walls were made permeable

to plasmid DNA by treatment with $CaCl_2$ and resistance to Ampicillin and Tetracycline was used to select transformants. This vector, with a size of about 300bp, confers Ampicillin resistance on its host. The transformed bacterial stock was kept at -20°C in 40% glycerol.

Plasmid extraction: Extraction of plasmid DNA (pUCJ probe) from small volumes of bacterial cell cultures was performed following the protocol of Birnboim & Doly (1979) with a few modifications. Transformed bacteria were streaked out on agar plates containing Ampicillin to obtain single colonies. An individual clone was grown in 4ml of Luria broth (Bacto-tryptone/Bacto-yeast extract/NaCl adjusted to pH 7.5 with NaOH) overnight at 37°C.

The extraction of plasmid DNA is based on selective alkaline denaturation of the high molecular weight chromosomal DNA of the bacteria while the covalently closed circular plasmid DNA remains double-stranded. Upon neutralization, chromosomal DNA renatures to form an insoluble clot, leaving plasmid DNA in the supernatant (Birnboim & Doly 1979).

After overnight incubation at 37°C, 3ml of culture was transferred by two successive 1 min. centrifugations into a 1.5ml microcentrifuge tube. The supernatant was subsequently removed by aspiration, with a fine tip. Glycerol (40%) was added to the remainder and stored at -20°C. The pellet was frozen at -70°C for 30 min. after which it was thawed. The pellet was subsequently resuspended in 100ul of the lysozyme solution (lysozyme/glucose/EDTA/Tris-HCl, pH 8.0) and incubated at 0°C for 30 min. to achieve the breakdown of the bacterial cell walls.

After the incubation, 200ul of the alkaline-SDS solution (NaOH/SDS) was added and the tube was gently vortexed to complete the lysis of the bacterial cell walls. The tube was maintained for 5 min. at $0^{o}C$ and 150ul of the high salt solution (potassium acetate/HOAc or NaAc, pH 4.8) was added. The contents of the tube were gently mixed by inversion for a few seconds, during which time a clump of DNA formed. The tube was then maintained at $0^{o}C$ for 60 min. to allow most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. The tube was then centrifuged for 5 min. at $0^{o}C$ and 0.4ml of the clear supernatant was transferred to another tube. Thereupon 200ul of phenol (saturated with 0.1M Tris-HCl, pH 8.0) was added and the tube was vortexed. Then 200ul of chloroform : isoamyl alcohol (24:1) was added and the tube was vortexed again, and centrifuged for 1 minute. The top phase (0.4ml) was collected and transferred to another tube. RNAse A in Tris-HCl (10ul of 1mg/ml), pH8.0 was added and incubated at $37^{o}C$ for at least 30 minutes. The phenol/$CHCl_3$ extraction was repeated. Ethanol (95%, 1ml) was added and the DNA allowed to precipitate for 2 min. at room temperature. The precipitate was collected by centrifugation for 2 min. and the supernatant removed by aspiration overnight at $-20^{o}C$. The DNA pellet was dissolved in 100ul of a solution of sodium acetate and Tris-HCl, and reprecipitated with 2 volumes of ethanol overnight after which the supernatant was removed by aspiration. It was then washed with 0.5 - 1ml 70% ethanol, centrifiged for 15 min. and the supernatant removed. Finally the DNA was air-dried and resuspended in 30 - 50ul $T_{10}E_1$, depending on the size of the pellet.

Determination of the pUCJ probe concentration and purity: This

was done in a similar way as for the rabbit DNA by means of a spectrophotometer and agarose gel electrophoresis in the presence of ethidium bromide. According to these results the DNA was diluted conservatively to a final volume of 1ug/ul.

Restriction endonuclease digestion of pUCJ probe: The procedure followed was as described previously for the rabbit DNA, with the exception that a double digest of DNA was necessary. The two restriction enzymes used to excise the insert from the pUC18 vector were HindIII and EcoRI (Boehringer Mannheim Biochemicals); their recognition sequences flank the insertion site.

Gel electrophoresis of pUCJ probe: A 20cm, 1% low melting temperature agarose gel, without Ethidium Bromide was used to separate the digested DNA in 1x TAE (Tris-base/Glacial acetic acid/EDTA) buffer. Before loading, 0.1ul Bromophenol Blue was added to the samples. Molecular weight markers were run on either side of the gel. Electrophoresis was performed overnight at 30V and at 4^{o}C. Two successive washes of the gel in distilled water with 0.2ug/ml Ethidium Bromide were carried out the next morning. The digested DNA was visualized on the transilluminator and the smaller fragment ($\pm$320bp) cut out as thinly and quickly as possible. It was transferred to a microcentrifuge tube and melted at 65^{o}C. An equal volume of sterile distilled water was added, the tube vortexed and stored at -20^{o}C (Feinberg & Vogelstein 1983). This was the stock of pUCJ probe used for pUCJ DNA hybridization.

pUCJ DNA hybridization:

Oligolabeling: The DNA fragment in agarose was thawed and melted. The total reaction volume was 50ul. The protocol outlined by Feinberg & Vogelstein (1983) was followed with a few modifications. The probe (30ul) was added to 4.5ul of sterile distilled water, boiled for 5 - 10 min. and immediately cooled to $0^{o}C$ for 30 seconds. Thereafter 10ul of 5x Oligolabeling buffer (a mixture of hexanucleotide primers/Hepes/ß-Mercaptoethanol/$MgCl_2$/ Tris) was added, as well as 50uCi radioactive deoxycytosine triphosphate (α^{32}PdCTP) and 5U Klenow (the large fragment of DNA polymerase I). The tube was centrifuged (1 sec.) and incubated overnight at $37^{o}C$. Hereby the process of endlabeling was completed.

Spermine precipitation: Unincorporated nucleotides were removed from the pUCJ probe solution by spermine precipitation. The probe solution was brought to a final volume of 500ul with TE^{-4} (Tris/EDTA). To this 8ul of Salmon sperm DNA (SSDNA 10mg/ml) and 8ul of spermine (tetrahydrochloride, 100mM), which is responsible for the precipitation, were added. After it was mixed it was cooled to $0^{o}C$ for 5 - 10 min. and then centrifuged at $4^{o}C$ for 10 minutes. From the supernatant 2ul was added to scintillation fluid and the remainder removed by aspiration. To the pellet 400ul of 0.5M NaCl in TE^{-4}, a buffer in which the pellet is dissolved, and 40ul NaOH (4N), for denaturization were added. The tube was incubated at $37^{o}C$ for two successive periods of 15 min. with a mixing of the solution in between to dissolve the DNA completely. After incubation 120ul Tris (2M, pH 7.5/7.6), to neutralize the DNA, was added and the solution mixed very well.

Of the incorporated fluid 2ul was also added to scintillation fluid. The probe was left on ice until hybridization on the same day.

Both the percentage incorporation and the total incorporated counts were determined by means of a liquid scintillation analyser (Hewlett-Packard). A minimum of 1 x 10^6 counts $minute^{-1}$ of hybridization solution was required before addition of the probe to the membrane.

Hybridization: Membranes were prehybridized in prewarmed (65^oC) prehybridization solution (SSPE ($NaCl/NaH_2PO_4.H_2O$/EDTA adjusted to pH 7.7 with NaOH)/Denhards/SDS). Before prehybridization, 500ul SSDNA (1mg/ml) was boiled for 5 min., cooled at 0^oC for 5 min. and added to 15 ml prehybridization solution. The membrane was incubated overnight at 65^oC. The inclusion of SSDNA was crucial to limit background hybridization on the membrane (Feinberg & Vogelstein 1983). After denaturation the probe was added to the prehybridization solution in the bag which was resealed and incubated overnight at 65^oC.

The hybridization was followed by variable stringency washes. The stringency of the washes increased with a decrease in the salt concentration and an increase in temperature. Membranes were successively washed at the different stringencies until Geiger counting of the membranes indicated suitable levels of ^{32}P on the membranes. Two successive washes in wash number one (2x SSPE/0.1%SDS) were performed for 10 min. each at room temperature and one wash in wash number two (1x SSPE/0.1% SDS) at 46^oC for 5 min. before suitable levels of ^{32}P on the membranes were

obtained.

Autoradiography: Fingerprints were visualized by exposing the membranes to X-ray film (Fuji) at -70^{o}C between two intensifying screens for periods up to 10 days. Photographs were then taken of the exposed X-ray film.

Analysis:

Since bands from lanes on more than one membrane had to be compared, a mathematical procedure was used. Each gel was run with at least three marker lanes. Least-squares regression constants were calculated for each marker lane, relating the distance moved by marker bands in that lane to their molecular weights. Log/log transformation of the data was necessary to get a good fit which yielded r^2-values with minimum value 0.978. All rabbit DNA lanes had marker lanes on both sides but not always adjacent. An interpolation for the regression constants for the lanes to the left and to the right of a rabbit DNA lane yielded regression constants which related the mobility of each band in a rabbit DNA lane to its molecular size.

All visible lanes on the fingerprints were scored by measuring their migration in mm from the origin (Westneat 1990). The mobilities of all the bands were then converted to molecular sizes, using the regression constants described above.

Determination of the independence between the SNAP and pUCJ probes: The extent of non-independent bands (overlap) between the two probes was calculated by determining a standard error of the

measurement of the bands. Four different fragments were measured 30 times and the standard deviation as well as the standard error of the mean for each band were calculated. The molecular weights of all the fragments were converted to mobilities (mm), using the already calculated regression constants. 95% confidence limits around these mobilities were constructed by adding and subtracting twice the standard error of the mean from all mobilities. Thereafter, the mobilities were converted back to molecular weights to determine the range of each specific band. The fragments produced by the two probes were compared for all individuals to determine the number of identical bands between the two probes used. Histograms of the dispersion of the fragments were drawn for both probes and for the overlapping bands. These frequencies were then compared using a Kolmogorov-Smirnov two-sample test.

Analysis of rabbit fingerprints: A computer programme was written (Appendix A) which compared molecular sizes of each band in one lane with the bands of all the other lanes. When comparing bands from different samples, various degrees of difference between two bands were considered as representing a match between the two bands. Degrees of difference in molecular weights tested were 1% - 5% of the molecular size of the band with which the other bands were compared. In this way, a match interval around each band was determined. These differences in molecular weights are reflected by the differences in mobility between the bands of different samples (Figure 4). If more than one band of a compared sample were included in the match interval around a particular band, two different ways of finding a match were used: In the first method the largest band inside the degree of difference was considered

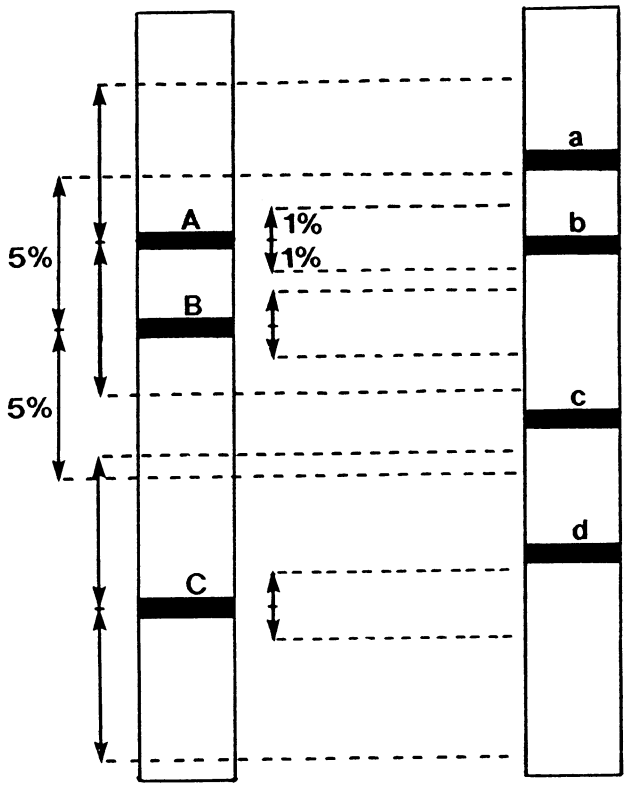

FIGURE 4

A diagrammatic representation of the degrees of difference in molecular weight reflected by mobility (1 - 5%) of bands that were taken into account in finding a match and an illustration of the two different methods that were used in finding a match between bands.

1% Mobility, 1st method: Matched bands = Ab

5% Mobility, 1st method: Matched bands = Aa, Bb, Cd

1% Mobility, 2nd method: Matched bands = Ab

5% Mobility, 2nd method: Matched bands = Ab, Bc, Cd

to be the match. In the second method the band with a molecular size closest to that of the band with which it was compared was considered to be the match (Figure 4). A stringency of 3% of the molecular size of the band that the other bands were compared with yielded computer-generated results that closely resembled those obtained when visually inspecting the samples. Dissimilarity between samples (representing individual rabbits) was calculated as the number of bands not shared by the two individuals as a fraction of the combined number of bands observed for the two individuals. This, multiplied by 100, resulted in a percentage difference (PD) value for each pairwise comparison (Gilbert *et al.* 1990, Yuhki & O'Brien 1990). The average percentage difference (APD) among all the individuals for each of the two probes used, was also calculated and a mean average percentage difference (MAPD) for the two probes used (Gilbert *et al.* 1990, Yuhki & O'Brien 1990). The PD values, expressed as fractions, were used to construct UPGMA dendrograms summarising the degrees of dissimilarity between the individual rabbits. UPGMA dendrograms were constructed for each one of the two different probes used as well as for the two probes combined.

RESULTS

Figure 5 presents the photographs of the fingerprints for the domestic rabbits with the SNAP and pUCJ probes and figures 6, 7A and 7B, 8 those of the riverine rabbits for the same two probes respectively. Figures 7B and 8 were a combination of fingerprints of autoradiographs that were exposed for different lengths of time for up to 10 days.

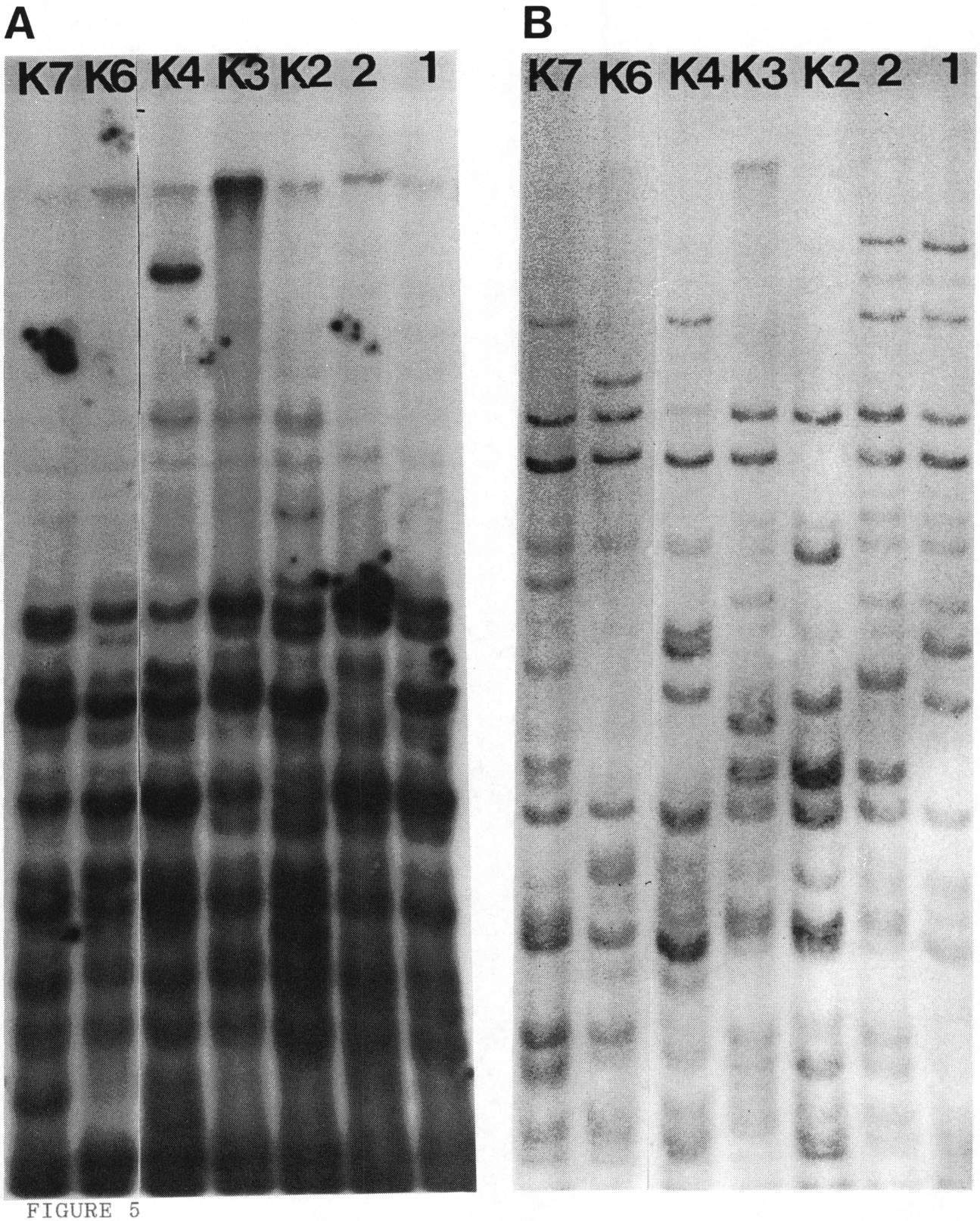

FIGURE 5

Fingerprints of domestic rabbits with two different probes:

A: pUCJ probe, B: SNAP probe

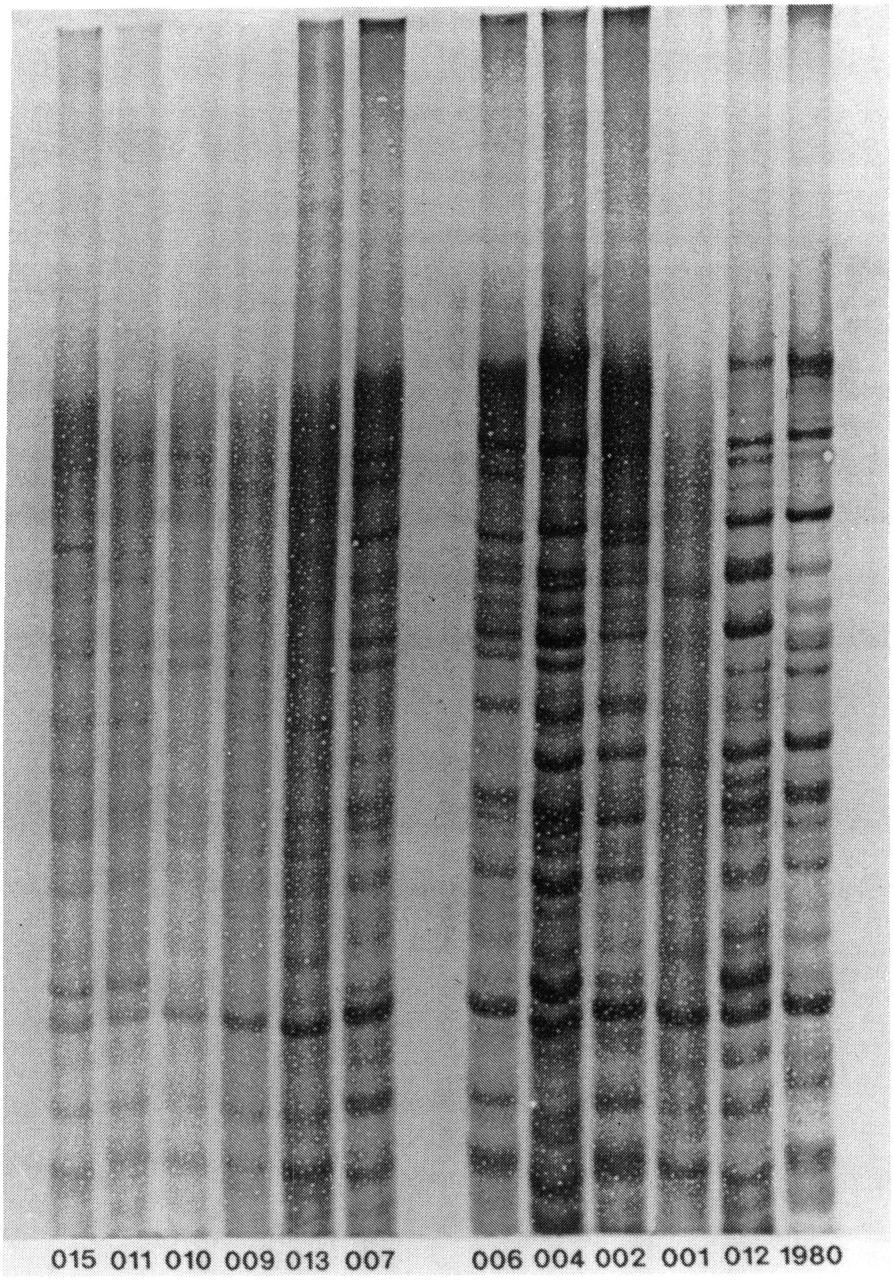

FIGURE 6

Fingerprints for the riverine rabbits probed with the SNAP probe. Empty lanes represent the marker lanes.

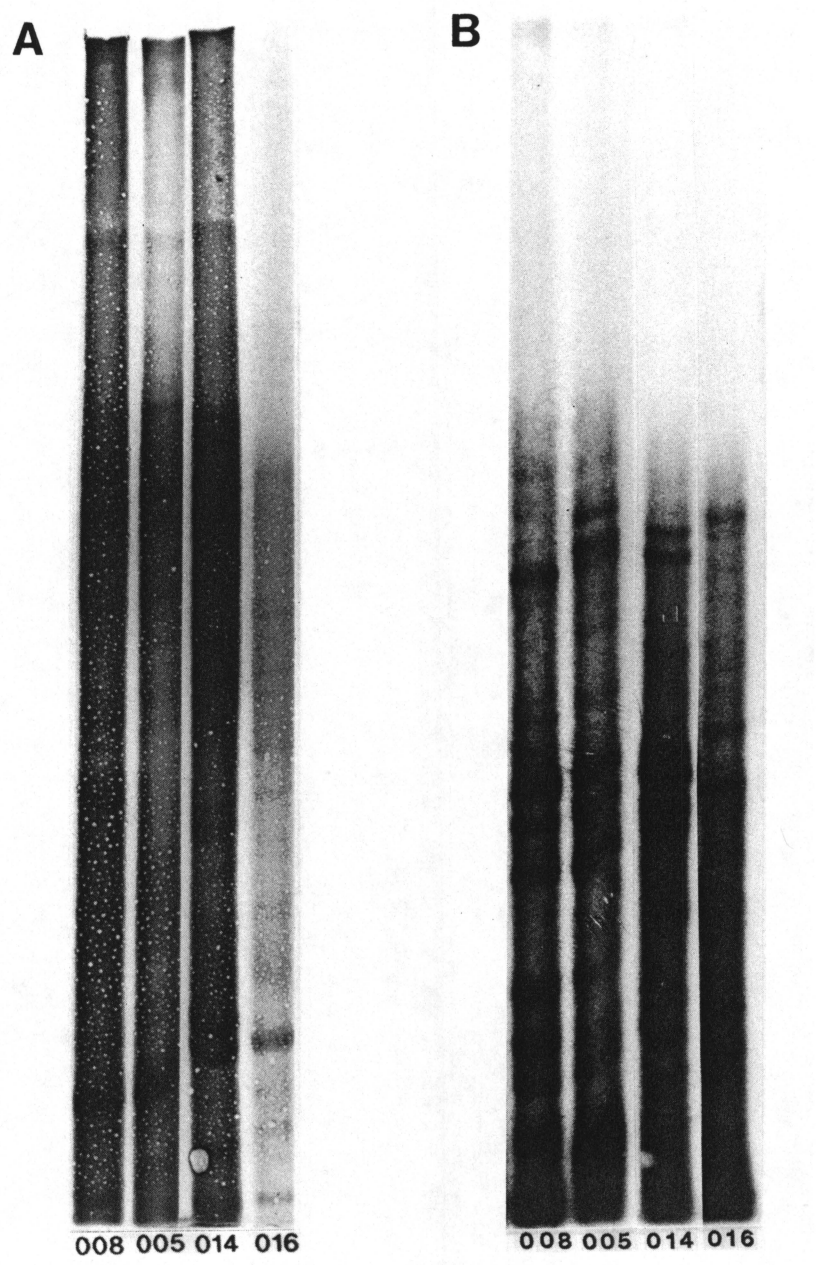

FIGURE 7

Fingerprints of the riverine rabbits probed with the SNAP (A) and pUCJ (B) probes.

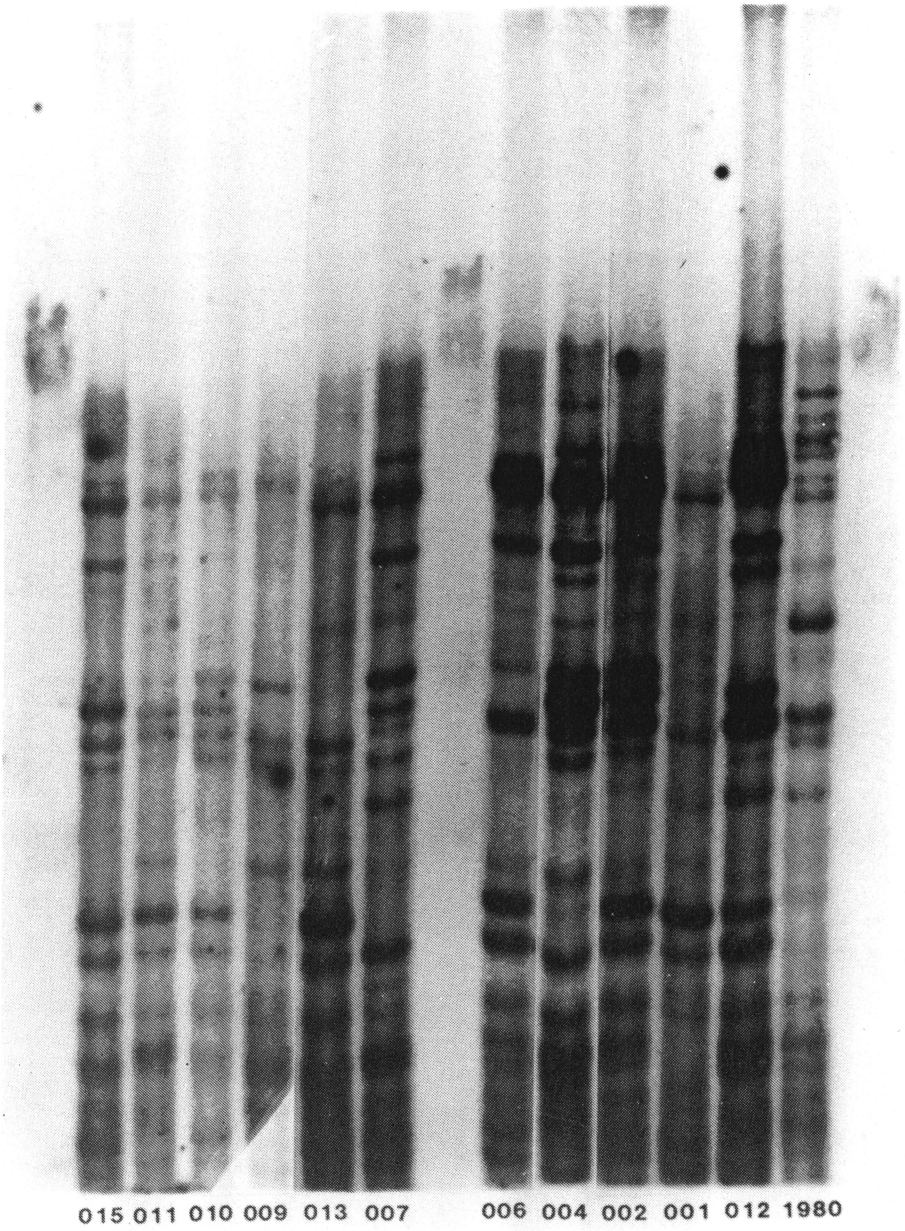

FIGURE 8

Fingerprints of the riverine rabbits probed with the pUCJ probe. Empty lanes represent the marker lanes.

Independence of the SNAP and pUCJ probes: The mean standard error for the measurement of the bands was 0.05mm resulting in a 95% confidence interval of $\pm$0.1mm. After this was taken into consideration 98 overlapping bands were found between the two probes. Histograms (Figures 9, 10 & 11) illustrate the dispersion of the fragments for the two probes and for the overlapping fragments between these probes. For all three comparisons no significant differences were found at a 5% level of significance. SNAP & pUCJ (Kolmogorov-Smirnov X^2 large sample approximation = 2.99, df = 2, $0.2 < p < 0.3$), SNAP & overlap (X^2 = 1.31, df = 2, $0.5 < p < 0.7$) and pUCJ & overlap (X^2 = 2.26, df = 2, $0.3 < p < 0.5$).

The number of fragments observed: The total number of fragments observed for the domestic rabbits varied between 26 and 30 (Table 5). The number of fragments observed per individual riverine rabbit varied between 15 fragments for specimen 005 and 32 fragments for specimen 1980 with the SNAP probe, whereas 16 fragments for specimen 005 and 28 fragments for animals 004 and 009 were observed with the pUCJ probe. This resulted in a total number of fragments which varied from 31 fragments for 005 and 59 fragments for animals 004 and 1980 (Table 6).

Inter-individual fingerprint variation: Both methods of defining a match, as described in the materials and methods, yielded virtually identical results. The method where a fragment was matched with the fragment closest to it in molecular weight was used throughout the analysis.

For the riverine rabbits the PD values for the SNAP probe ranged

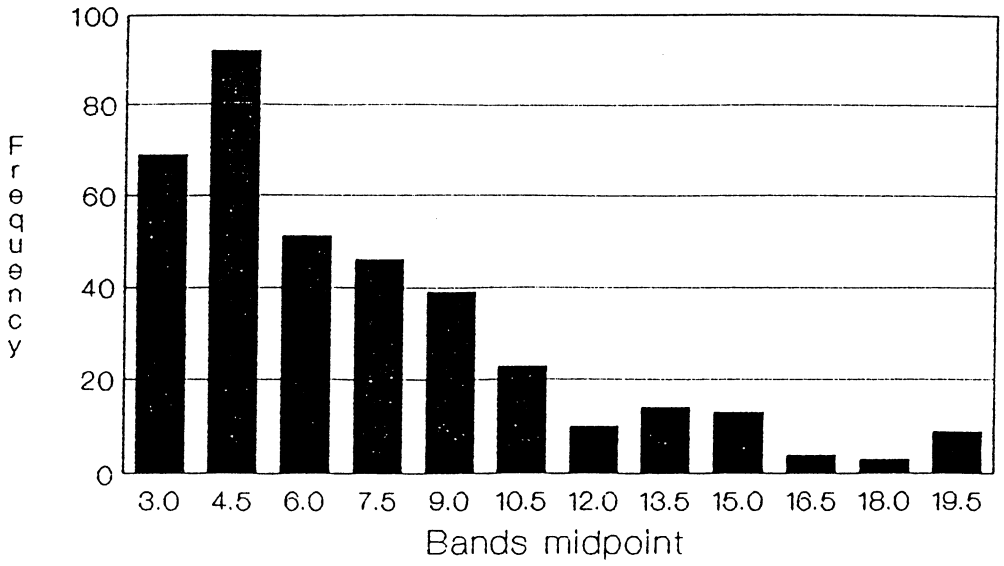

FIGURE 9
Frequency of the distribution of different sized bands using the SNAP probe.

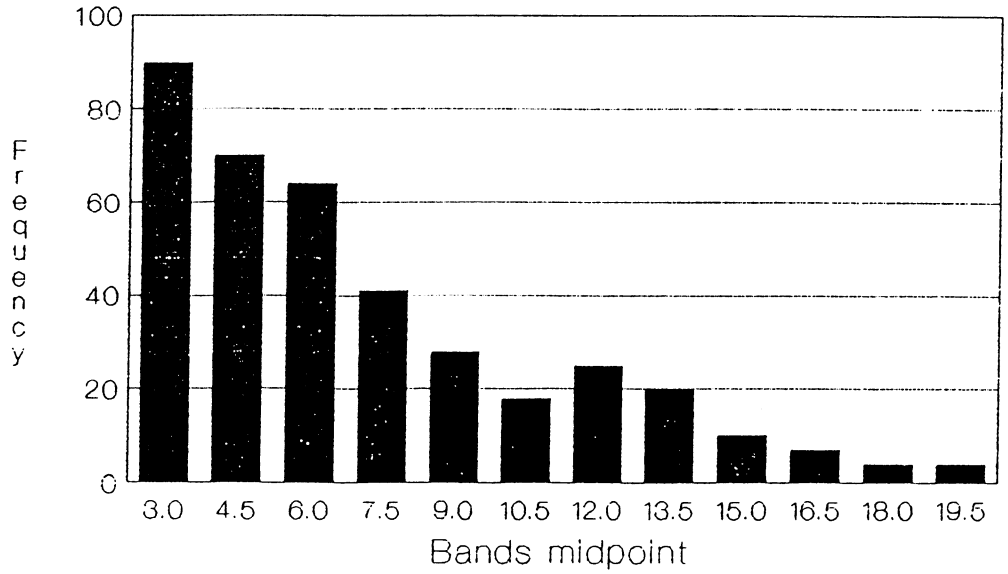

FIGURE 10
Frequency of the distribution of different sized bands using the pUCJ probe.

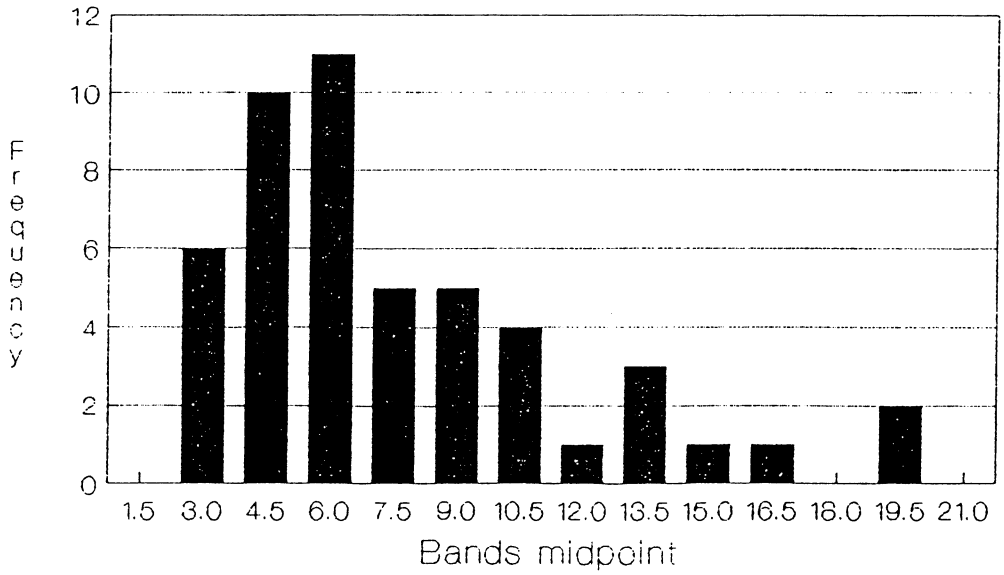

FIGURE 11
Frequency of different sized overlapping bands between SNAP and pUCJ probes.

TABLE 5

The number of fragments observed with the different probes for the domesticated rabbits.

		NUMBER OF FRAGMENTS USING PROBE:		
RABBIT ID	SEX	SNAP	pUCJ	TOTAL
1	F	14	12	26
2	M	16	13	29
K2	F	10	20	30
K3	F	15	12	27
K4	M	12	15	27
K6	F	11	15	26
K7	M	16	14	30
Mean		13.4	14.4	27.9
s.e.m.		0.92	1.04	0.67

TABLE 6

The number of fragments observed with the different probes for the riverine rabbits.

		NUMBER OF FRAGMENTS USING PROBE:		
RABBIT ID	SEX	SNAP	pUCJ	TOTAL
001	F	18	21	39
002	M	24	25	49
004	F	31	28	59
005	F	15	16	31
006	M	28	27	55
007	F	25	26	51
008	M	18	20	38
009	F	20	28	48
010	M	21	22	43
011	F	21	25	46
012	M	30	26	56
013	M	25	23	48
014	M	19	20	39
015	M	24	22	46
016	F	22	25	47
1980	M	32	27	59
Mean		23.3	23.8	47.1
s.e.m.		1.25	0.85	1.98

from 10.7% to 62.2%, and those of the pUCJ probe from 7.4% to 61.1%. The corresponding APD values were 34.7% and 29.8% for the SNAP and pUCJ probes respectively. These are similar to the values obtained for domestic rabbits which ranged from 20.0% to 41.9% in the case of the SNAP probe and from 4% to 48.1% for the pUCJ probe. The respective APD values were 32.3% for the SNAP probe and 33.7% for the pUCJ probe. The MAPD for the riverine rabbits was 32.3% compared with that of 33% for the domestic rabbits (Table 7).

The PD values for the riverine rabbits were used to construct UPGMA representations of the relative dissimilarities among individuals (SNAP Figure 12A and pUCJ Figure 12B). Analysis involving stringencies varying between 1% and 5% yielded trees with very similar topologies for each of the two probes. The topologies of the UPGMA trees for the SNAP probe and for the pUCJ probe are also somewhat similar. Note the following clusters common to both trees: 10/11, 4/6 and 2/9. Individuals 5, 8, 1 and 15 also cluster separately in both analyses. It was decided to draw a tree for the combined PD values of the two probes in order to increase the number of fragments for each individual (Figure 12C). From this it appears that rabbit 5 is most different from the rest of the population, followed by 8 and 14. Individuals 8 and 14 also differ largely from each other.

The APD value for the group of rabbits comprised of individuals 1, 8, 14 and 15 is 46.6% for SNAP and 34.2% for pUCJ. The MAPD of these two values is 40.4% (Table 8). For a group of rabbits comprised of individuals 1, 2, 8 and 14 the APD was calculated as 46.5% for SNAP and 32.9% for pUCJ with a MAPD of 39.7% (Table 8).

TABLE 7

A comparison of PD, APD and MAPD values between the domestic and riverine rabbits.

SPECIES:	DOMESTIC RABBITS		RIVERINE RABBITS	
PROBE:	SNAP	pUCJ	SNAP	pUCJ
PD Range	20-41.9	4-48.1	10.7-62.2	7.4-61.1
APD	32.3	33.7	34.7	29.8
MAPD	33		32.3	

PD - percentage difference

APD - average percentage difference

MAPD - mean average percentage difference

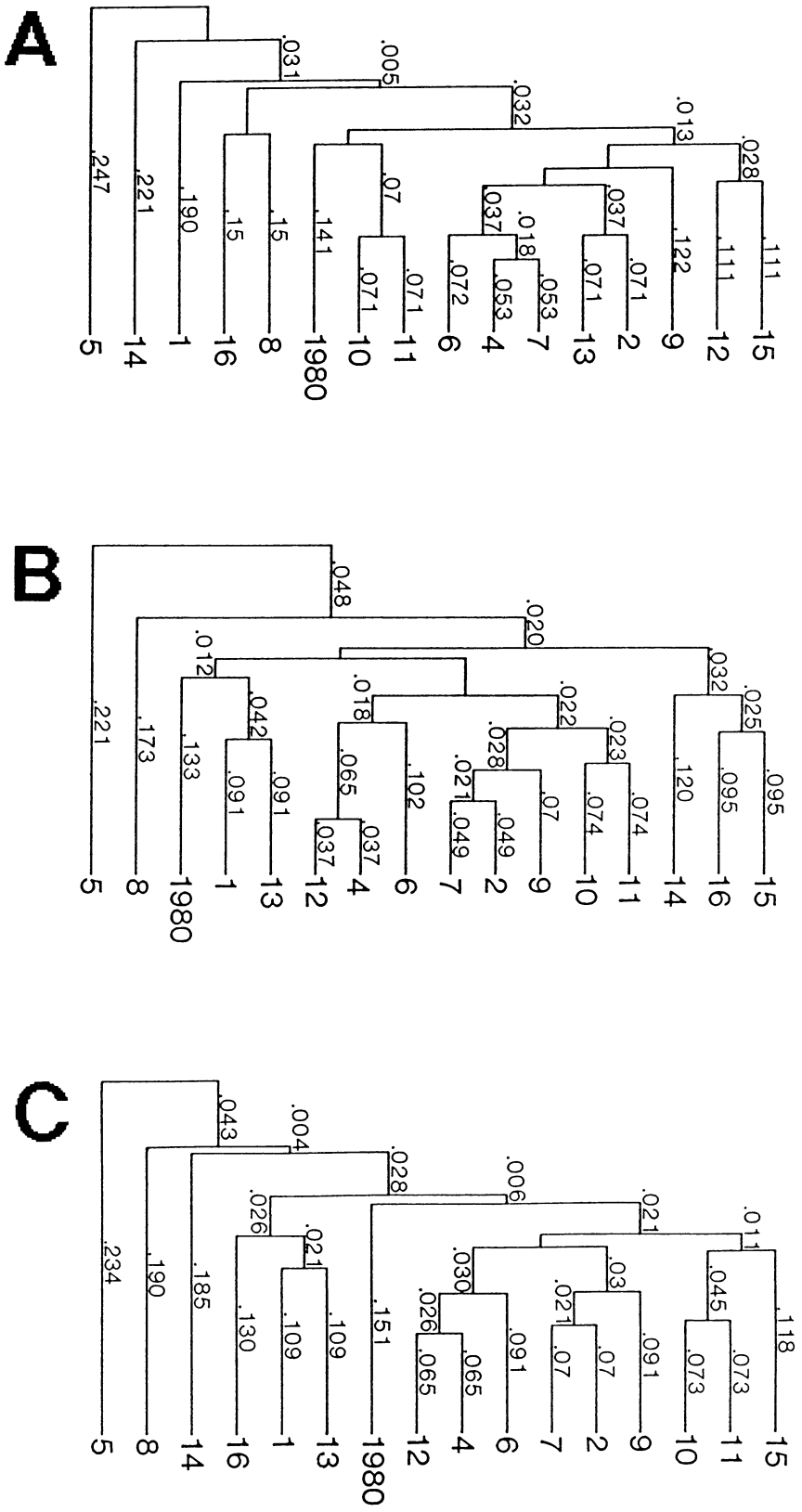

FIGURE 12

UPGMA representations of the relative dissimilarities among individuals for the PD values of the SNAP probe (A), pUCJ probe (B) and the combination of the two probes (C).

TABLE 8

The PD, APD and MAPD values for two groups of riverine rabbits.

GROUPS:	1, 8, 14 & 15		1, 2, 8 & 14	
PROBE:	SNAP	pUCJ	SNAP	pUCJ
PD Range	38.1-62.2	23.8-41.5	33.3-62.6	21.7-41.5
APD	46.6	34.2	46.5	32.9
MAPD	40.4		39.7	

PD - percentage difference

APD - average percentage difference

MAPD - mean average percentage difference

DISCUSSION

The genealogical relationships of the riverine rabbits were completely unknown. Consequently the fingerprints could not be used to construct a genealogy of the rabbits. The band sharing coefficients are very high: these can be calculated as twice the number of shared bands divided by the total number of bands in the fingerprints of two individuals being compared (Lynch 1990). The higher the average band sharing coefficient, the poorer are the confidence limits for positive paternity analysis (Wetton *et al.* 1987, Westneat 1990, Amos & Dover 1991). When the fragments are run further, the probability of band sharing is reduced since additional restriction fragments are resolved (Ely *et al.* 1991).

Independence of the SNAP and pUCJ probes: Lynch (1988) predicted a large standard error for estimates of similarity based on

relatively few fragments. Since the separate analyses for the SNAP and pUCJ probes are based on some 20 to 30 fragments per individual, most of the differences between them could be attributed to sampling error. This is evident from the fact that a large number of the branch lengths of the UPGMA trees for the two separate probes are not statistically significant. Therefore the two sets of data were combined as an attempt to increase sample size and reduce the standard error of the estimates. However, before they could be combined it was necessary to ensure that the two probes were independent.

If the repeat sequences of SNAP and pUCJ probes were not independent, both probes would have been expected to bind to a larger proportion of the large and medium sized fragments than to the small fragments. If the two types of VNTRs were linked in some way, it would be expected that large restriction fragments will include binding sites for both the probes more often than the small restriction fragments. It was, however, statistically determined that there was no significant difference in the distribution of the overlapping fragments among the different size classes when compared with one another. This indicates that the SNAP and pUCJ probes can be treated as independent probes and that the data for the two probes can be combined to increase the number of fragments for each individual.

The number of fragments observed: The number of fragments observed per individual on the fingerprints of the domestic rabbits is fewer than that of the riverine rabbits. This is because the fingerprints of the domestic rabbits were produced during part of the experimental phase and conditions were less

than optimal, resulting in lower resolution of the fingerprints. Consequently the smaller fragments on the bottom 2cm of the gel, which could not be separated clearly into different fragments were not scored.

The combined number of fragments observed per individual on the DNA fingerprints of the riverine rabbits varied between 31 and 59 in total. The number of fragments observed was affected by the purity of the DNA, since the DNA extract of the individual with the least bands (005), contained a large amount of protein due to rapid clotting of the blood sample immediately after collection.

The APD for the captive riverine rabbit population was 32.3% compared to a figure of 33% for the domestic rabbits. For two reasons these figures indicate that the captive riverine rabbits are relatively inbred. First, the domestic rabbits used are known to be inbred, even though the exact amount of inbreeding is not known for the colonies sampled. The APD value for the domestic rabbits corresponds to that for the riverine rabbits. Secondly the mean APD of the captive riverine rabbit population is significantly smaller than that quoted for outbred, wild populations (70 - 90%) by Gilbert *et al.* (1990). APD values cannot be translated directly into levels of homozygosity since DNA fingerprinting technology assays the noncoding part of the genome, a genetic component that probably has very different characteristics compared with coding DNA. Gilbert *et al.* (1990) and Amos & Dover (1991) indicate, however, that high homozygosity correlates with low dissimilarity between DNA fingerprints. Westneat (1990) also found that relatives have more fragments in common than non-relatives. Therefore low APD values might be

strongly suggestive, but not definitive, of inbreeding among the riverine rabbits.

Few of the branch lengths of the UPGMA trees constructed with the PD values for the riverine rabbits were statistically significant. This suggests that a larger number of probe/enzyme combinations are needed to arrive at statistically meaningful trees. However, two factors lead to confidence in the basic topologies of these trees: first, the occurrence of similar UPGMA topologies for each of the two probes with analyses involving stringencies varying between 1% and 5%, and secondly the similarity of the UPGMA trees for the SNAP and pUCJ probes and the occurrence of clusters common to both trees.

From these trees it appears that rabbit 005 is most distinct from the rest of the population but, as indicated above, this sample contained protein impurities. It is therefore suspected that this factor contributed to the large difference between 005 and the rest of the population and that this may be an artifact of the molecular analysis. There is also a large difference between rabbits 001, 008 and 014 and the rest of the group, but because individuals 001 and 014 did not contain protein impurities these values are accepted as a true reflection of their position relative to the rest of the population.

In order to determine if the inbreeding had any obvious effects on the animals, and to be able to be more specific about the animals which can be used as founders in a breeding programme, it was decided to make an assessment of the sperm quality of the riverine rabbits in captivity.

CHAPTER FOUR

SPERM COLLECTION AND COUNTING

INTRODUCTION

Inbreeding depression, arising from a loss of heterozygosity, undermines the reproductive physiology of individuals (Crow & Kimura 1970, Ralls *et al.* 1983, Crow 1986, Gilpin & Soulè 1986). This is observed in a high frequency of spermatozoan abnormalities in the ejaculates, a lower concentration of spermatozoa (sperm) and a lower motility of the sperm of the inbred animals (O'Brien *et al.* 1986). In contrast to the uniformity of shape of normal sperm, abnormal sperm of inbred strains display a spectrum of morphological variations (Wyrobek 1979). Examples of animals where such variations have been found are the cheetah *Acinonyx jubatus* (O'Brien *et al.* 1983, O' Brien *et al.* 1985, O'Brien *et al.* 1986, O'Brien *et al.* 1987) and the lion *Panthera leo* (Wildt, Bush, Goodrowe, Packer, Pusey, Brown, Joslin & O'Brien 1987, Brown, Bush, Packer, Pusey, Monfort, O'Brien, Janssen & Wildt 1991, Packer, Pusey *et al.* 1991).

Spermatozoan abnormalities can be detected when examining permanent preparations of smears stained with nigrosin-eosin where the sperm are stationary, readily visible, lie flat and are in a good optical medium (Beatty 1970). A distinction can then be made between eosinophilic and non-eosinophilic sperm, which enables a 'live/dead' count and a detailed examination of morphology to be made on the same slide (Beatty 1970, Dott & Foster 1972). One of the best predictors of the fertility of

semen is the proportion of unstained spermatozoa (Beatty 1970). Eosinophilic sperm have permeable membranes and when collected as an ejaculate these can be considered dead.

The value of a differential 'live/dead' stain in assessing the quality of semen and in evaluating male fertility is considerably reduced if conditions, particularly the time and temperature of staining, are not rigidly controlled (Hancock 1951, Campbell, Dott & Glover 1956). However, Dott & Foster (1975) found that the proportion of eosinophilic spermatozoa on an eosin-nigrosin smear was the same as in the original semen if a sample was fixed and kept at room temperature for 48h. Furthermore, an increase of eosinophilic spermatozoa only occurred if stored at 40^{o}C for more than 48h (Dott & Foster 1975).

In an attempt to determine the sperm viability of the captive riverine rabbits (*Bunolagus monticularis*), a 'live/dead' count was made and the sperm were examined for any abnormalities.

MATERIALS AND METHODS

The riverine rabbits at De Wildt were captured on November 7th 1991 to collect semen from the males by electroejaculation with a rectal probe. Unfortunately, the probe was too large and no semen could be obtained but all the animals were weighed. On December 20th 1991 they were recaptured and semen was collected whereafter all the rabbits were weighed to obtain an estimate of their body condition. The procedure was repeated on January 22nd 1992.

Electroejaculation was performed by using a square wave

stimulator set to 50 cycles.sec.$^{-1}$. A stimulus of between 10 and 20V was applied for approximately 3 seconds. The stimulus was repeated several times after the animal had relaxed. Semen (about 15ul) was usually produced during relaxation following the first stimulus and was collected in a funnel. It was then withdrawn from the funnel with a pipette and placed into a tube. A small drop of semen was used to make a smear on a slide which was immediately examined under a microscope on a hot stage (37^{o}C) for assessing the motility of the sperm. The rest of the semen was fixed by dilution with 0.1% formalin-saline (200ul) (Dott & Foster 1975). The spermatozoa were stained later that day with nigrosin-eosin (Dott & Foster 1972, 1975) by adding 50ul of semen solution to 50ul of nigrosin-eosin (10% nigrosin and 0.6% eosin Y) (Dott & Skinner 1989) and leaving it for 5 min. at room temperature. Thereafter, one drop was used to make a smear on a slide which was left to dry. Two slides were made for every individual. When mounting a cover slip, Entenel was used as a mountant whereafter the slides were left to dry for 24h at 37^{o}C.

The slides were examined under a microscope and the proportion of stained and unstained spermatozoa estimated. The unstained sperm were classified as normal sperm, abnormal sperm and spermatozoa with a proximal droplet. Although species-specific, the proximal droplet gives an indication of incomplete maturation of the sperm.

RESULTS

During the December capture semen was only obtained from six of the seven males (no semen obtained from individual 014). The

semen was examined under the microscope on the hot stage and sperm was found in the ejaculate of only one of the six males (individual 002). The proportion motility could not be determined because most of the spermatozoa were obscured by prostatic granules.

During the January capture, semen was obtained from only four of the males, no semen being obtained from males 008, 013 and 014. Once again, the proportion motility could not been estimated because of the presence of prostatic granules.

Examination of the permanent preparations revealed that only male 002 produced sperm on both occasions when semen was collected. Interestingly, 92% of the sperm were eosinophilic while of the remainder, 4% were normal and 3% abnormal because of a bend or broken tail or neck and only 1% had a proximal drop. Figure 13 presents the eosinophilic and non-eosinophilic sperm of male 002.

Although all the rabbits appeared to be in a good condition during all three of the captures, there was a general tendency for body weights to decrease from the first to the third capture (Table 9).

DISCUSSION

There are several possible explanations for why only one male produced sperm. The first is that the riverine rabbit may be a seasonal breeder although Duthie (1989) considered this to be unlikely. Although he found no signs of breeding during June, July or October through to January in the Karoo, and although the

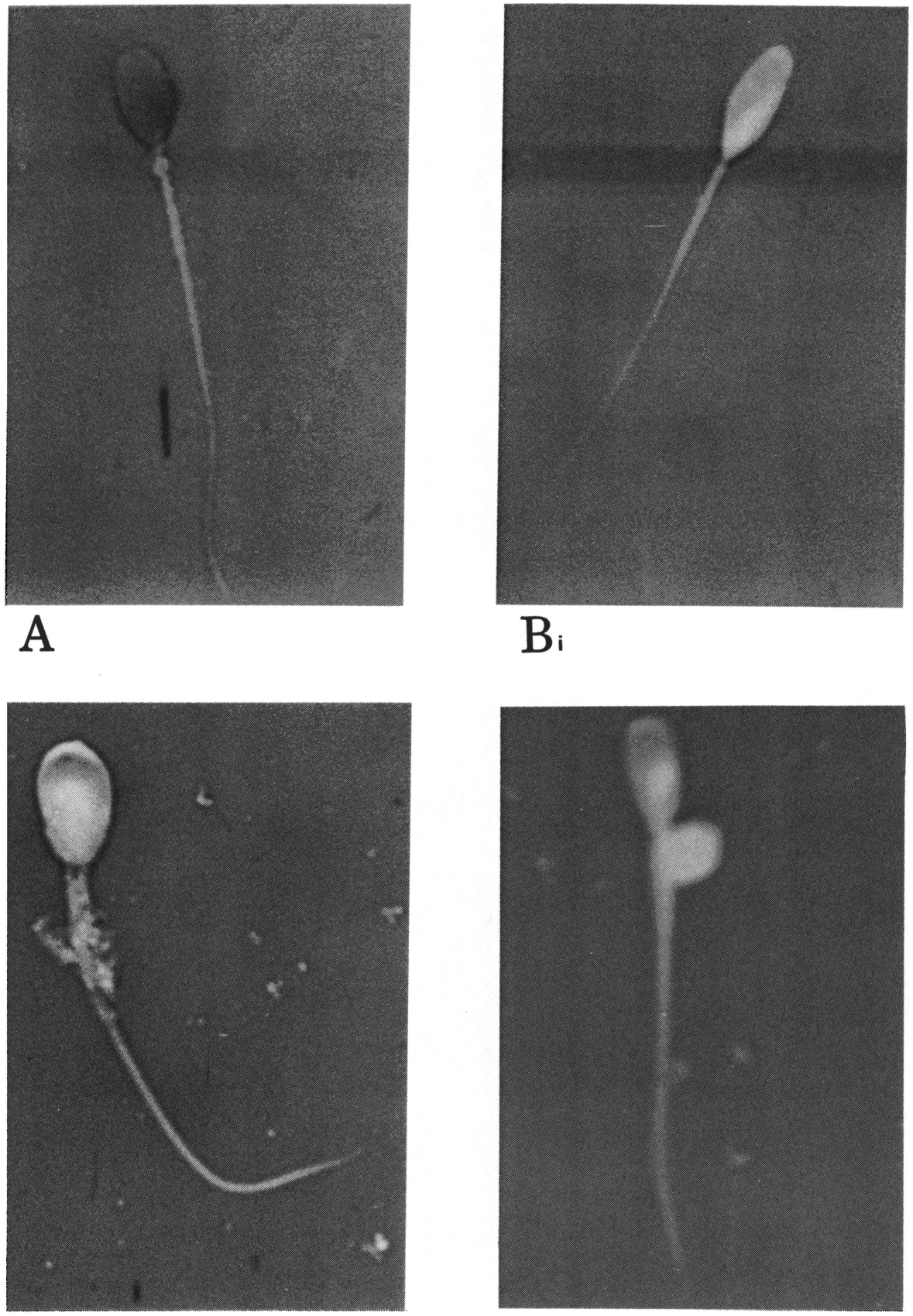

FIGURE 13

Eosinophilic (A) and non-eosinophilic (B) sperm of male 002. Non-eosinophilic sperm were divided into normal (Bi), abnormal (Bii) and with a proximal droplet (Biii).

TABLE 9

The body weights of the riverine rabbits on the days of semen collection.

RABBITS	SEX	WEIGHTS		
		NOV	DEC	JAN
001	F	1.57	1.55	1.57
002	M	1.44	1.42	1.35
004	F	1.70	1.62	1.63
005	F	1.48	1.47	1.38
006	M	1.39	1.38	1.34
007	F	1.68	1.675	1.59
008	M	1.42	1.38	1.39
012	M	1.52	1.48	1.50
013	M	1.47	1.45	1.38
014	M	1.57	1.52	1.47
015	M	1.36	1.36	1.32
016	F	1.67	1.43	1.47
Mean		1.52	1.48	1.45
s.e.m.		0.034	0.028	0.030

breeding period appeared to extend from August to September and from April to May, he nevertheless suggested that riverine rabbits might be aseasonal breeders (Duthie 1989). At De Wildt, these rabbits were observed to breed during the winter months (Van Dyk pers. comm.*). It might thus be possible that they are seasonal breeders at De Wildt, in the Transvaal, but not in the Karoo, due to the differences in climatic conditions in these two areas and the captive conditions pertaining to De Wildt.

The breeding season can be defined as the length of time between the first fertile coitions of the season and the time when the last young of the season are capable of independent existence (Haugen 1942). A definite breeding season was found for cottontail rabbits (Beule & Studholme 1942, Haugen 1942), domestic rabbits (Cooke 1981, Daly 1981a, Garson 1981, Parer & Fullagar 1986) and hares (*Lepus americanus, L. europaeus, L. arcticus, Lepus* spp.) (Keith 1981). The breeding season may be a response to weather conditions which may also act indirectly by influencing food availibility and energy demands (Cooke 1981, Daly 1981a, Garson 1981, Keith 1981, Parer & Fullagar 1986), or it may be a response to daylength (Keith 1981) which is dependent on latitude. Under a relatively constant daylight on the Equator, *Lepus europaeus* breeds year-round (Flux 1969). Seasonality seems to be more pronounced in regions with large fluctuations in climate such as the temperate regions, because of the large seasonal variations in environmental conditions of rabbits. However, the fact that the rabbit and hare are induced ovulators (Rowlands & Weir 1984) must also be taken into account.

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* VAN DYK, A. (personal communication). De Wildt Cheetah Breeding Facility, De Wildt.

Weather conditions influence food availability which in turn influences breeding season. Since the riverine rabbits at De Wildt are provided with a constant source of food, it is unlikely that the difference in weather conditions between the Karoo and De Wildt could have had a major effect on the breeding season of these animals. However, the difference in latitude and the corresponding difference in daylength may have had such an effect. If riverine rabbits are seasonal breeders, spermatogenesis in all but the one male from which sperm were obtained may have ceased. Evidence exists for the cessation of or decline in spermatogenesis at the end of the breeding season in domestic rabbits (Garson 1981, Glover, D'Occhio & Millar 1990). There is, however, also evidence that in some species, the males continue to produce sperm after the breeding season has ended and the testes have regressed (de Reviers, Courtens, Courot & de Reviers 1990, Glover *et al.* 1990). Sampling of the riverine rabbits throughout the year is thus necessary before any final conclusions can be made.

The second explanation for why sperm was only obtained from one male is that the results obtained were influenced by the technique and/or equipment used. This is unlikely, because of the consistency of the results. Spermatozoa were obtained from the same individual (002) on both occasions and no semen was obtained from the same individual (014) on both occasions.

An alternative explanation is that only one of the seven males is fertile due to inbreeding depression as a result of long term inbreeding. Due to the lack of knowledge regarding the riverine rabbits in the Karoo, especially regarding their genetic

variation, it is impossible to be more definite about the level of inbreeding in the rabbits at De Wildt. The DNA fingerprinting results (Chapter 3) suggest that they are indeed inbred, although the degree of inbreeding is unknown.

Sperm were collected as an ejaculate and it is therefore likely that the eosinophilic sperm were all dead. If this was so, then only 5% of the sperm of rabbit 002 were alive and normal. This value is much lower than that obtained for the inbred cheetahs, for which 29% of the sperm had no morphological abnormalities (O'Brien *et al.* 1983, O'Brien *et al.* 1985, O'Brien *et al.* 1986, O'Brien *et al.* 1987, Wildt *et al.* 1987). The low value of normal live sperm found for the riverine rabbit may also indicate inbreeding depression in a similar way. However, these are only tentative findings and more collections of semen should be performed in order to draw more meaningful conclusions.

In order to relate possible inbreeding of the De Wildt population of riverine rabbits to that of the population in the Karoo, it was attempted to estimate the average heterozygosity of the latter population.

CHAPTER FIVE

AVERAGE HETEROZYGOSITY AND GENE FLOW IN THE KAROO POPULATION

The aim of this chapter is to speculate on the average heterozygosity and inbreeding of the riverine rabbit population in the Karoo. To accomplish this, factors affecting their geographic range are firstly considered and secondly the population fragmentation is discussed as well as whether dispersal or migration can take place between demes or subpopulations and the implications of this. This knowledge allows speculation of the effective population size and average heterozygosity.

Distribution and abundance of riverine rabbits:

Riverine rabbits are confined to the magisterial districts of Sutherland, Frasersburg, Carnarvon, Loxton, Beaufort West and Victoria West in the central Karoo (Van Rensburg, Robinson & Skinner 1979) (Figure 14). These areas comprise three different veld types (Acocks 1988) - Central Upper Karoo (27), Western Mountain Karoo (28) and a small section of the False Arid Karoo (35). Several arguments indicate that the rabbits do not occur outside this geographic area:

1. The area lies on an isolated plateau of 1200 - 1500m above sea level which extends to the east into the False Upper Karoo (36) (Figure 14).
2. The climate changes from tropical to temperate along the western boundary of the False Upper Karoo (Schulze 1947). The False Upper Karoo falls in a region with a ratio of precipitation

FIGURE 14

A map of the distribution of the riverine rabbit in the central Karoo.

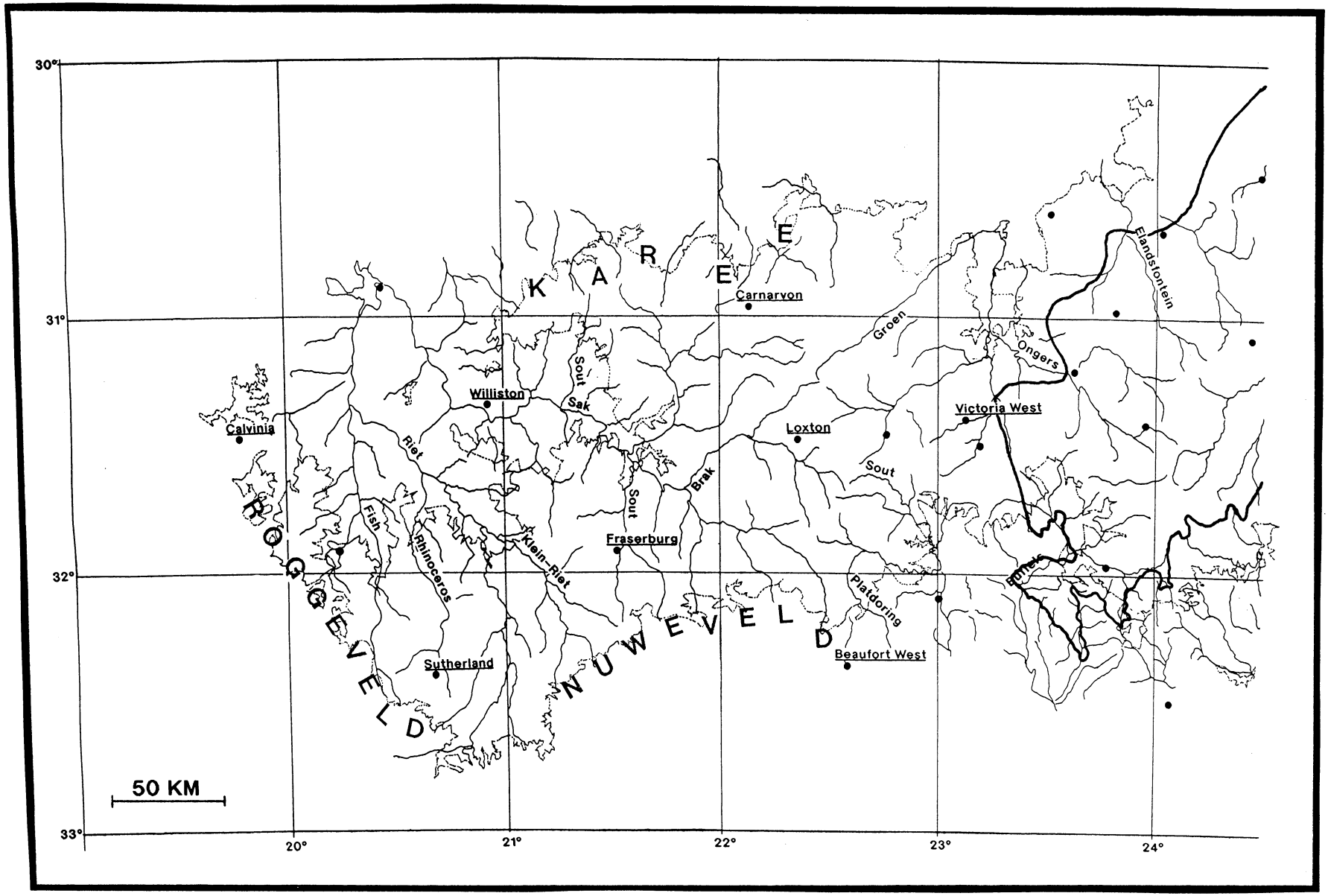
A: Rabbits present

B: Suitable habitat, rabbits absent,

C: Habitat destroyed

------ 1200m contour

——— Border between False Upper Karoo in the east and the rest of the area where the rabbits occur.



to temperature of 21 - 40, while the Central Upper Karoo and the Western Mountain Karoo fall in a region where the value of this ratio is from 11 - 20 (Schulze 1947).

3. The vegetation in the False Upper Karoo does not correspond to that in the area where the rabbits occur. *Pentzia incana* and *Salsola* spp, which occur in the riverine scrub (Duthie 1989), also occur in the False Upper Karoo. However, the remainder of the plants are quite different. It therefore seems unlikely that riverine rabbits occur in the latter vegetation type.

4. Intensive searches for riverine rabbits indicate that they do not occur in the False Upper Karoo (Van Rensburg *et al.* 1979, Duthie 1989) east of their present geographic range (Figure 14).

A comparison of the riverine rabbit's present distribution with their known past distribution (Robinson 1981a) shows a 50% reduction in range over the last 40 to 50 years (Duthie 1989, Duthie *et al.* 1989). This decrease is due mainly to a destruction of approximately two-thirds of the original habitat for purposes of cultivation (Robinson 1981a,b). Suitable habitat along the banks of the Fish and Rhinoceros rivers was removed in an attempt to turn these rivers into the 'bread basket of the Cape' (Green 1955). However, this attempt failed due to a shortage of irrigation water (Duthie *et al.* 1989).

The literature reveals several factors that affect lagomorph densities and these could contribute to the observed low densities of riverine rabbits. Some of these factors may also explain the absence of *Bunolagus monticularis* from areas where suitable habitat occurs, such as the banks of the Ongers River (Duthie 1989). It is possible that the riverine rabbits occurred

in these areas, but became locally extinct and have not yet recolonized. Some of the factors affecting lagomorph densities are climatic factors, predation, parasites and other lagomorphs (Angerbjorn 1981, Cooke 1981, Flux 1981, Gibb 1981, Häkkinen & Jokinen 1981, Keith 1981).

Climatic conditions: A population crash due to climatic conditions such as a drought period or a snowstorm was reported for rabbits, *Oryctolagus cuniculus* (Cooke 1981, Daly 1981a, Gibb 1981) as well as hares, *Lepus* spp. (Angerbjorn 1981, Häkkinen & Jokinen 1981, Keith 1981). Such severe climatic conditions may result in a food shortage, weakening the animals and consequently making them more vulnerable to diseases and predation (Gibb 1981, Häkkinen & Jokinen 1981). Heterogeneity of the environment appears to buffer the population against irregular changes in weather patterns (Daly 1981a). Rabbit numbers decrease when only highly fibrous foods are available and successful breeding may occur when rabbits have foods containing about 12% protein (Cooke 1981). Duthie (1989) found that the intake of *Pteronia erythtocaetha*, *Kochia pubescens* and *Salsola glabrescens* increased during the drier winter months. The percentage fibre content of these species is, however, unknown. Other species of *Salsola* contained 15 - 18% fibre (Steenkamp & Hayward 1979), but it is not known how this compares with *Salsola glabrescens*. It is also unknown whether the fibre content of the three plant species taken more during winter is relatively high compared with the fibre content of the rest of their intake, but it compares favourably with the fibre content of 40% of foods which rabbits were seldom observed to eat even during drought periods (Cooke 1981). It has been suggested that weather patterns in the Karoo

follow a six year cycle, in which three wet years are followed by three dry years (Davies pers. comm.*). A severe drought in the Karoo National Park during 1987 resulted in a population crash in the red rock rabbit (*Pronolagus* spp.) and the hyrax (*Procavia capensis*) (Davies pers. comm.*) and it is likely that the riverine rabbit population was similarly affected during a drought period. Subsequent floods reduced population numbers of red rock rabbits and hyraxes still further (Davies pers comm.*). The six year cycle in weather patterns is not very clear when looking at the rainfall data for 1980 - 1990 of the relevant towns in the central Karoo (Weather Bureau 1991). However, it is possible to distinguish between the very dry and very wet seasons (Table 10). It is possible that the collapse of farm dams, which may happen quite often, together with flooding and destruction of the rabbit's habitat comprising riverine scrub vegetation has had drastic effects on riverine rabbit numbers in these areas (Van Rensburg *et al.* 1979).

Predation: Although the extent of predation on the riverine rabbits is unknown, it is suggested that the weakening of lagomorphs due to a shortage of food because of climatic conditions can make them much more vulnerable to predation and thus cause a decline in population numbers (Gibb 1981, Häkkinen & Jokinen 1981).

Parasites: Although the riverine rabbits in captivity are covered with fleas, the importance of parasitic infection of riverine rabbits in the Karoo is not clear, but this may also increase

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* DAVIES, R.A.G. (personal communication). Mammal Research Institute, University of Pretoria, Pretoria.

when the animals are weakened by food stress due to climatic factors. This will result in more rabbits taken as prey and more rabbits dying, which will also reduce the population numbers (Angerbjorn 1981, Gibb 1981, Häkkinen & Jokinen 1981).

Other lagomorphs: There is increasing evidence that the distribution or abundance of one species of lagomorph may be affected by that of other hares or rabbits (Keith 1981). It was speculated that arctic hares might be excluded from remaining Scottish forests through the presence of European hares (Keith 1981). It was also found that *Lepus timidus* increased in numbers in Finland as *L. europaeus* became scarce, and vice versa (Flux 1981). In the Karoo 88% of the riverine rabbit distribution coincides with that of either *Lepus saxatilis* or *Lepus capensis* or both (Van Rensburg *et al.* 1979), which may have a influence on the population numbers of the riverine rabbit (Robinson 1981a).

Population fragmentation of riverine rabbits:

Following distribution surveys conducted during 1979 (Van Rensburg *et al.* 1979) and 1985 (Duthie 1989) it was apparent that *Bunolagus monticularis* had a linear but disjunct distribution in a small part of the central Karoo. This pattern of distribution has some consequences. Following the distribution in 1979 (Van Rensburg *et al.* 1979) the population can be divided into at least two subpopulations as a result of habitat destruction. A small subpopulation occurs west of the Fish river while a larger subpopulation is found east of the Rhinoceros river (Figure 14). Information about their current distribution is unavailable, but it is likely that their distribution is less continuous than in

TABLE 10

The total rainfall(mm) of the yearly season (July - June) of the driest and wettest years (1980 - 1990) of the towns in the vacinity of the distributional area of *Bunolagus* in the central Karoo from west to east.

TOWN	DROUGHT		FLOOD	
	mm	season	mm	season
Calvinia	158.4	1983/1984	260.8	1984/1985
	150.1	1986/1987	318.6	1985/1986
Sutherland	219.1	1983/1984	400.1	1984/1985
	234.6	1986/1987	419.2	1985/1986
Williston	86.1	1983/1984	235.8	1987/1988
	98.8	1984/1985	305.9	1988/1989
	89.3	1986/1987		
Fraserburg	141.8	1982/1983	284.5	1980/1981
	123.4	1986/1987	288.0	1985/1986
Carnavon	52.2	1980/1981	313.6	1987/1988
	123.4	1986/1987	314.1	1988/1989
Loxton	85.9	1982/1983	383.3	1987/1988
	141.4	1986/1987	340.8	1988/1989
Beaufort West	Station closed - incomplete data			
Victoria West	159.0	1984/1985	384.7	1985/1986
	121.5	1986/1987	546.4	1987/1988

1979 as a result of various factors, including changes in climatic conditions affecting the species habitat and human interference resulting in the destruction of habitat. It is thus likely that there are more than two subpopulations. The division of a population into subpopulations has important implications in terms of population genetics.

Population subdivision: A population whose range is composed of more or less geographically isolated patches, interconnected by patterns of gene flow, extinction, and recolonization, forms a metapopulation or a population of subpopulations. Such a population can be considered approximately panmictic if the separate colonies exchange in the order of one or more migrants per generation. The general effect of population subdivision is to increase the total genetic variance in the total metapopulation, but at the same time to decrease the variation within subpopulations. Although this can have favourable effects, it can lead to inbreeding within the subpopulations or within the total population if it is too pronounced (Li 1978, Hedrick, Brussard, Allendorf, Beardmore & Orzack 1986, Lande & Barrowclough 1987).

Inbreeding may be counteracted by postnatal dispersal (Ralls, Harvey & Lyles 1986). Dispersal can also be described as migration resulting in movement of successful breeding individuals or gametes from one subpopulation to another (Hartl 1980, Chambers 1983). Low amounts of migration (approximately one individual per generation) will maintain the presence of all the genetic variation of a species in all the subpopulations, but will allow genetic differentiation among subpopulations in

response to local selective pressures (Kimura & Ohta 1971, Allendorf 1983). Dispersal has beneficial effects in maintaining both the effective population size and average heterozygosity, especially if the population has undergone a degree of inbreeding (Frankel 1983). The subpopulations may exchange individuals more often with their neighbouring subpopulations than with distant ones, so that the immigrants do not constitute a random sample of the entire population. This will result in neighbouring subpopulations having similar gene frequencies due to the continuous interchange of individuals between them (Li 1978). The effects of population subdivision can be measured quantitatively by the fixation index (F_{ST}), which is an estimate of allele frequency divergence among demes (Allendorf 1983, Hartl 1987).

The long-term persistence of metapopulations depends upon a balance between the frequent extinctions of subpopulations and recolonizations of newly suitable patches of habitat or areas where the earlier subpopulation has become extinct (Lande & Barrowclough 1987). However, if colonization occurred by a single founder female or a pair of founders, the potential exists for the resulting subpopulation to lose the initial levels of genetic diversity present in the species (Namkoong 1983) due to inbreeding and genetic drift.

Since the riverine scrub occurs only on the banks of the rivers, and there are many interconnecting non-perrenial rivers, it would appear that their distribution might be continuous enabling rabbits to disperse along these rivers to neighbouring subpopulations. This would promote gene flow among neighbouring

subpopulations. Pronounced differences in gene frequencies may however exist between the ends of this continuous population. However, their distribution is more likely to be disjunct along the banks of the rivers which will make dispersal from one deme to another almost impossible (Robinson pers. comm.*) and will result in subpopulations which are genetically very similar within the subpopulation but genetically rather different among subpopulations.

Dispersion and migration:

An estimate of the importance of migration requires knowledge of dispersion of riverine rabbits. Presently there is nothing known about their dispersion, thus it is necessary to infer riverine rabbit dispersion from data for other species of rabbits and hares. Although the riverine rabbit is classified as a rabbit (Robinson 1981b, Robinson & Skinner 1983), many of its behavioural patterns correspond with those of hares. It is therefore necessary to consider the behavioural patterns of both rabbits and hares.

Domestic Rabbits: Studies on the domestic rabbit (*Oryctolagus cuniculus*) have shown this species to be group living in a system of underground warrens (Cowan & Garson 1985, Bell 1986, Cowan & Bell 1986, Cowan 1987a,b). They have a gestation period of 27 - 30 days after which the altricial young are born and raised underground (Cowan & Bell 1986). Major dispersal events of juveniles, both male and female, were observed seasonally,

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* ROBINSON, T.J. (personal communication). Mammal Research Institute, University of Pretoria, Pretoria.

particularly towards the end of the breeding season (Daly 1981a,b, Parer 1982). Males and females were equally represented among dispersing rabbits less than 60 days old. However, after 60 days of age about twice as many males as females had dispersed (Garson 1981, Parer 1982, Parer & Fullagar 1986). Kittens were quite likely to move over long distances and some dispersed more than 600m (Daly 1981a, Parer & Fullagar 1986).

Cottontails: *Sylvilagus* spp. are solitary and adopt intermediate behavioural patterns between underground nesting and repeated dispersal from a surface nest, raising their young above ground and having a longer gestation period (37 - 43 days) than underground nesting species (Trent & Rongstad 1974, Verts & Carraway 1981, Cowan & Bell 1986). No significant difference in movement distances between sexes or ages were found for the cottontail rabbits *Sylvilagus floridanus* (Scribner & Warren 1990). The eyes of *Sylvilagus floridanus* open about seven days after birth (Beule & Studholme 1942, Haugen 1942). The kittens of *Sylvilagus transitionalis* leave the nest permanently at 16 days of age, after which they establish sheltering forms in which two and three youngsters will sometimes nest together (Tefft & Chapman 1983). Dispersion of a male individual of *Sylvilagus floridanus* was found to be 1372m in 220 days (Bothma 1969).

Hares: Hares are solitary nocturnal herbivores. They do not utilise underground burrows but only forms which are depressions in the ground or vegetation (Flux 1981). The leverets are born precocial after a gestation period of 37 to 50 days (Cowan & Bell 1986). They disperse to new forms three days after birth, but still come to the mother for very short daily feeding periods

(Röngstadt & Tester 1971, Flux 1981). Further dispersion may result from other influences for example, the population is at or near the carrying capacity and individuals must either disperse or die (Wolff 1981).

Riverine Rabbits: *Bunolagus monticularis* is a solitary, nocturnal rabbit which utilises forms and below ground breeding stops and which has a gestation period of 35 to 36 days. They produce a small litter of altricial kittens whose eyes only open at seven days and locomotion develops at 10 days. These kittens leave the nest permanently at 14 days of age (Duthie 1989). This behaviour corresponds well with that of cottontails. It can thus be suggested that there would not be any significant differences between dispersal distances of the different sexes and ages. If the group living domestic rabbits disperse more than 600m and the cottontail rabbits 1372m it can be expected that the riverine rabbits are also able to disperse over distances in the order of a kilometre.

If this holds and riverine rabbits occur in a continuous population, migration of rabbits among closely neighbouring demes is possible if the habitat is continuous. Consequently a continuity of gene frequencies is expected, with the possibility of relatively large differences between widely separated demes. Some of the genetic variation in the population would therefore be distributed among the demes. The magnitude of such spatial heterogeneity will depend on the local deme size and the total geographical extent of the metapopulation (Lande & Barrowclough 1987). Alternatively, if the population is divided into only a few subpopulations separated by unsuitable habitat, it is highly

unlikely that migration can take place between subpopulations, which may be separated by 10km and more. This implies that much of the genetic variance will be distributed among rather than within subpopulations (Lande & Barrowclough 1987) and although total genetic heterogeneity may be relatively high, each subpopulation will probably be relatively homogeneous due to inbreeding within subpopulations.

A surprising result of the fingerprints (Chapter 3) was that the wild-captured rabbit clustered inside the dendrogram for the captive colony. This indicated large genetic similarity between the 1980 individual and the captive rabbits. However, this was not completely unexpected taking into account that all the rabbits, including the 1980 individual, can be traced back to the same farm near Victoria West. It is possible that the 1980 individual fortuitously belonged to the same ancestral family group as the others. In a large outbreeding population of rabbits it would have been expected that the 1980 sample would constitute an outgroup, when compared with a captive inbred population. This expectation arose from the fact that a random individual taken from a population should differ largely from a group of captive, inbred rabbits arising from only four individuals of that subpopulation, if the original population was panmictic. Although DNA from only one wild-captured rabbit is available it appears that the local subpopulation or deme from which all these rabbits came is genetically rather homogeneous, resulting in similar DNA fingerprints when different individuals were compared. If this were true for the deme at Klipbank, then it is likely to be true for all the demes of riverine rabbits scattered along the river banks of the central Karoo, the evidence leads to the suspicion

that relatively little gene flow takes place among demes of riverine rabbits.

Since the latter is probably true for the riverine rabbits in the Karoo, it might be expected that the subpopulations are relatively inbred which make them even more susceptible to extinction due to stochastic events (Chapter 1) (Shaffer 1981, Gilpin & Soulé 1986).

Effective population size

For estimating the heterozygosity of the Karoo population of riverine rabbits it is necessary to estimate the effective population size.

Duthie (1989) and Duthie *et al.* (1989) estimated on the basis of home range sizes that the extant habitat could possibly support 1435 rabbits, but varying population densities and their absence from some apparently suitable habitat suggests that the real population size is much lower.

The effective population size (N_e) can be defined as the number of individuals in an ideal population in which all individuals breed and which would have the same genetic properties (in terms of random genetic drift, heterozygosity and distribution of allele frequencies) as the actual population being considered (Wright 1939, Solbrig & Solbrig 1979, Chambers 1983, Hedrick 1984, Allendorf 1986, Lande & Barrowclough 1987, Lande 1988). The ratio of effective population size to census population size varies greatly for natural populations and the effective

population number is less than the census number (Chambers 1983).

The following factors affect the effective population number (Crow & Kimura 1970, Ewens 1979, Solbrig & Solbrig 1979, Soulé 1980, Gilpin & Soulé 1986, Crow & Denniston 1988):

Uneven contribution of gametes by different individuals in the population: If the individuals of a population do not have the same expected number of progeny, the effective number will be less than the census number (Crow & Kimura 1970). Inbreeding depression results in the decrease of an individual's reproductive ability (Senner 1980, Falconer 1981, Selander 1983, Soulé 1986). It is possible that some rabbits are more inbred than others, resulting in an uneven contribution of gametes by different individuals. Mortality of the young is also responsible for an uneven contribution of gametes by different individuals. A decrease in effective population number will thus occur.

Inequality of the numbers of males and females contributing to the population: Unequal numbers of males and females contribute to a decrease in the effective size within a generation (Lande & Barrowclough 1987). Currently there is no reason to believe that there is any deviation from a 50:50 sex ratio in the Karoo riverine rabbit population, and consequently this factor is not expected to have an influence on the effective population size.

Population size fluctuations from generation to generation: A population is made up of a mixture of breeders and nonbreeders, and among the breeders, reproductive success varies with age and

size and therefore fluctuations will decrease the population size (Solbrig & Solbrig 1979). This factor probably affects the effective population numbers of riverine rabbits, since it is likely that population size fluctuates from generation to generation due to the previously mentioned factors, climatic conditions, predation, parasites, other lagomorphs and human influences.

Nonrandom breeding: Social structures e.g. harems, leks or other polygynous systems are responsible for a decrease in the effective size (Soulé 1980). Due to the fact that *Bunolagus monticularis* is a solitary species (Duthie 1989), there is no evidence that the reproduction of some individuals is suppressed by conspecifics. Therefore this factor appears not to contribute to a decrease in effective population size.

Subdivision of the population into subpopulations: Fragmentation with turnover of subpopulations has profound negative consequences for effective population size, potentially reducing effective population sizes by orders of magnitude compared to the actual total census count (Maruyama & Kimura 1980). This factor is likely to have a major impact on the effective population size of the riverine rabbit. Widespread (or increasing) habitat destruction has fragmented the population into subpopulations, without a likelihood of migration between subpopulations. The division of the population into at least two subpopulation is discussed further on in the chapter. Evidence for genetic similarity within demes was found in the fingerprints of the riverine rabbits.

Overlapping generations: Overlapping generations may also decrease the effective number of breeding adults (Soulé 1980). The riverine rabbit definitely has overlapping generations (Duthie 1989) and therefore the effective population size will be lower.

The factors affecting the effective population size are intertwined and mutually affect each other. An increase or decrease of one factor can greatly affect others and may consequently profoundly influence effective population size.

Average Heterozygosity

The average heterozygosity of a population can be defined as the average proportion of heterozygous individuals per locus and is a measure of genetic variation (Ayala 1982). Table 11 indicates the expected average heterozygosity ($m = 4N_e u$, $u = 10^{-6}$; Fuerst, Chakraborty & Nei 1977) for riverine rabbits in the Karoo for different possible effective population sizes. Estimates of heterozygosity represent equilibrium values reached after a period of time. With an upper limit effective population size of 2000 rabbits, the estimated heterozygosity is 0.08 (Table 11). If the extant habitat can support 1400 rabbits (Duthie 1989), the maximum effective population size will probably be closer to 1000, due to the influence of the factors affecting the effective population size, earlier discussed. The estimated heterozygosity of an effective population of 1000 individuals is 0.04 (Table 11). The most likely effective population size at the moment is between 300 and 800, but probably closer to 300 (Duthie pers.

comm.*). If an effective population size of 500 is assumed, a heterozygosity of 0.02 (Table 11) is expected.

Current knowledge suggests that the rabbits west of the Fish River constitute a discrete subpopulation, separated from the rest of the riverine rabbit population by a belt of destroyed habitat (Figure 14; Van Rensburg *et al.* 1979, Duthie *et al.* 1989). The area over which this subpopulation was found, suggests that the subpopulation size is probably below 100 individuals. If the N_e for this subpopulation is 100 and they were separated from the main population 30 generations ago which might have an effective population size of 1000 individuals with an average heterozygosity of 0.04, the average heterozygosity of this isolated subpopulation is estimated at 0.034 (Hartl & Clark 1989). This suggests that the western subpopulation has lost a significant amount of genetic variation due to genetic drift alone. Their low average heterozygosity, together with the fact that they are isolated from the rest of the population, jeopardise the survival of the subpopulation even more as a result of all the factors mentioned in Chapter One. In order to prevent this subpopulation from extinction it is necessary to consider active conservation of this subpopulation.

When comparing these estimated values for the riverine rabbits with the average heterozygosity of 0.051 for mammals (Ayala 1982), the values for N_e larger than 1000 compare favourably. However these values are unlikely to apply as explained

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* DUTHIE, A.G. (personal communication). Wildlife Society of Southern Africa, Johannesburg.

TABLE 11

Possible effective population sizes for the riverine rabbit population in the Karoo and the consequent estimated average heterozygosity ($m = 4N_e u$, $u = 10^{-6}$; Fuerst, Chakraborty & Nei 1977).

N_e	Heterozygosity
2000	0.08
1400	0.056
1000	0.04
700	0.028
500	0.02

previously and the more realistic value of around 0.02 is much reduced. These values, which could be even lower, may be indicative of inbreeding in the population or may reflect a bottleneck event (O'Brien & Evermann 1988). Alternative possible explanations may be behavioural disposition to assortative (consanguinous) mating or a species could evolve to an adaptive optimum for a particular environmental niche and then gradually shed its variability during an extended period of niche stability (O'Brien & Evermann 1988). However, of these three alternatives the first one is the most likely in the case of the riverine rabbit, because of their solitary nature and variable environment.

Due to the very low estimated values of the average heterozygosity of the population in the Karoo genetic management of these wild rabbits is very important and should be seriously considered.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

All the evidence leads to the tentative conclusion that the riverine rabbit population in the wild does not have a large amount of genetic variation. The number of riverine rabbits in the wild population in the Karoo may be small with an effective population number of some 500 individuals (Duthie pers. comm.*). The average heterozygosity for this effective population size was estimated at 0.02 (Chapter 5), significantly lower than the quoted average heterozygosity of mammals (Ayala 1982). In addition, at least one isolated subpopulation appears to exist west of the Fish River and the genetic variation in this subpopulation could be expected to be even lower than in the main population.

A number of authors believe that captive breeding programmes should attempt to maintain 90% of the genetic variation encountered in the wild over a period of some 200 years (Chesser, Smith & Lehr Brisbin 1980, Soulé *et al.* 1986) so that inbreeding depression in the captive population and the release of nonviable individuals into the wild can be prevented (Foose 1978). However, all species are not equally susceptible to inbreeding depression and some populations can adapt to inbreeding (Templeton & Read 1984), because the very occurrence of inbreeding creates selective conditions that favour those animals with genes or gene combinations that do well under

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* DUTHIE, A.G. (personal communication). Wildlife Society of Southern Africa, Johannesburg.

inbreeding (Templeton & Read 1984). However, this may have a negative influence on future adaptations to changes in the environment. Circumstantial evidence for the occurrence of inbreeding depression in the captive population of riverine rabbits was found (Chapters 3, 4).

The number of individuals required for the maintenance of fitness in a population depends on the definition and criteria for fitness, the intended duration of the programme, the generation time and the maximum tolerable loss of genetic variation (Soulé *et al.* 1986).

The first aspect to consider when initiating a breeding programme is short-term fitness i.e. the current viability and reproductive success of the individuals in the captive population (Templeton & Read 1984, Soulé *et al.* 1986). Even very modest decreases in heterozygosity because of genetic drift and/or inbreeding can reduce fitness (Soulé *et al.* 1986). Although species-specific, inbreeding depression may be avoided if the rate of inbreeding per generation remains below 2% (Soulé *et al.* 1986) and thus the minimizing of individual inbreeding coefficients should be a priority in captive breeding management (De Bois *et al.* 1990). The second aspect to consider is long-term fitness i.e. the capacity of the population to maintain itself indefinitely in its natural environment (Soulé *et al.* 1986). The retention of long-term fitness or viability is dependent on the preservation of genetic variation. Although populations can adapt to inbreeding (Templeton & Read 1984), inbreeding will definitely reduce a population's ability to survive in a natural, changing environment.

In order to preserve genetic variation it is prudent to have as many founder individuals as possible (Soulé *et al.* 1986, Hedrick & Miller in press). The reasons for this is: First, the more founder individuals, the larger the genetic variability of that generation and second, the larger the number of founders, the sooner the group will reach the target captive population size (Soulé *et al.* 1986). Genetic variation in captive populations can additionally be maximized by equalizing the founder individuals' genetic contribution to subsequent generations (Kimura & Crow 1963, Ely *et al.* 1991) and by maintaining isolated captive subpopulations homozygous for one allele and other subpopulations homozygous for alternative alleles (Chesser *et al.* 1980). The deleterious effects of genetic drift can be lessened to some degree by dividing the captive population into subpopulations (Chesser *et al.* 1980). To minimize the effects of inbreeding in these subpopulations it is however, necessary to optimize the effective population size and to exchange animals among the subpopulations (Chesser *et al.* 1980). However, care must be taken during such exchange in order that outbreeding depression, which is also species specific, is avoided. Outbreeding depression is a decrease in fitness which occurs when mating takes place between individuals of different inbred local subpopulations with little dispersal between subpopulations. The different local subpopulations may hypothetically have different coadapted gene complexes which confer high fitness upon the individuals bearing them, as long as the complexes remain intact. When matings occur between individuals with different coadapted complexes, recombination between the complexes occurs and individuals receiving recombinant complexes consequently suffer from lower fitness (Templeton & Read 1984). Outbreeding depression may also

be important with respect to translocations (the intentional release of animals to the wild; Griffith, Scott, Carpenter & Reed 1989). However, ill-considered translocations must be discouraged in order to protect a species from unnatural hybridization or introgression, and to conserve the genetic integrity of subspecies and ecotypes. Nevertheless, when a species as a whole is severely endangered translocations may be necessary for survival (Greig 1979) and this may be applicable to the riverine rabbit.

A breeding programme for the riverine rabbit was therefore established at De Wildt. Foose (1978) suggested that no matter how scrupulously such a genetic programme is applied it is possible, at best, to preserve only as much genetic diversity or variability as has been originally imported from the wild to found the captive population (Foose 1978). Since the extant captive population at De Wildt originated from only four rabbits from a single deme, the breeding programme started with little genetic variability. However, the fact that the founder number was low does not mean that attempts should be abandoned to promote the captive population because if the population size can increase quickly the effects of the bottleneck will be less profound (Hedrick & Miller in press).

Therefore a breeding programme was proposed (Appendix B) with a founder population size of 16 rabbits and in which rotation of the offspring takes place (Dippenaar 1989). When the captive population reaches a size of 64 individuals it should be divided into two discrete subpopulations with exchange of individuals between subpopulations, so that the final breeding population

size be at least 32 pairs for the whole captive breeding population. It was suggested that two wild-captured individuals are exchanged with two captive ones per generation until the breeding population size of 64 has been reached. Thereafter, excess rabbits can be released into the wild and the exchange rate can decrease to one animal per generation (Dippenaar 1989).

The present project used DNA fingerprinting to determine which of the existing 12 rabbits in captivity would be suitable for inclusion in such a breeding programme.

If a single animal has to be chosen for participation in a breeding programme and if fingerprint similarity of rabbits reflects inbreeding, specimen 005 would be the least inbred and would be the rabbit of choice (Chapter 3). However, because the number of fragments observed is affected by the purity of the DNA and since the DNA extract of this individual contained a large amount of protein this may be a spurious choice. Conservatively therefore, specimen 008 should be selected since it is the next most genetically distinct representative of the population (Figure 12C).

If a small number of rabbits with largely dissimilar fingerprints were to be chosen it would be 008, 014, 001 and any one of the remainder excluding 013 and 016. If founders for a breeding programme were to be chosen from the existing captive group of rabbits, 001, 008, 014 and 015 would be most suitable as is evident in a significantly higher MAPD value obtained for them (Table 8). This value is, however, still well below the APD values quoted for outbreeding species (Gilbert *et al.* 1990).

The finding that only one male, specimen 002, produced sperm makes this rabbit a definite choice for inclusion in a group of rabbits selected as founders in the proposed breeding programme. The MAPD value for the group of rabbits consisting of 001, 002, 008 and 014 is also higher than that of the whole population (Tables 7, 8) and this group would therefore also be a suitable choice. However, these rabbits are genetically relatively the most different from one another compared with the rest of the captive population, but they will be relatively similar to one another when compared with wild outbred rabbits.

An interesting result of the investigation was that the wild-captured rabbit, specimen 1980, clustered inside the dendrogram for the captive colony (Figure 12C). This indicates large genetic similarity between the wild-captured individual and the captive rabbits and suggests that the local population, or deme, from which all these specimens were derived is genetically rather homogeneous. If this were true for all the demes of riverine rabbits scattered along the river banks of the central Karoo, the capture of founders for a captive breeding programme would have to be performed over a wide range of the geographic distribution of riverine rabbits.

The above evidence also indicates that all the extant captive riverine rabbits are genetically similar since they arise from very few founders which all came from the same subpopulation which is itself probably rather inbred. In an attempt to estimate how inbred the extant captive riverine rabbits are, the possible minimum and maximum heterozygosities are determined. If the effective wild population size is 500 with a resulting

heterozygosity of 0.02 (Table 11), the heterozygosity of the captive rabbits could be as low as 0.006: this calculation assumes an effective population size of the captive rabbits of 4, implying a significant degree of inbreeding during three generations since 1987 if year old rabbits are able to reproduce. If, in contrast, the N_e of the wild population is 1000, resulting in a heterozygosity of 0.04 (Table 11), the heterozygosity of the captive population is unlikely to be higher than 0.034: this latter calculation assumes an effective population size of 10 for the captive rabbits and no inbreeding during three generations since 1987.

If any of the rabbits presently in captivity were to be used in a breeding programme with wild captured rabbits, it would be recommended that only a single one be used since the other captive rabbits are relatively similar to the one chosen.

If breeding has to start with a number of the extant captive rabbits, it is recommended that they are paired as follows: 015 and 007, 002 and 005, 014 and 016, 012 and 001, 008 and 004 and males 013 and 006 without females. It is also suggested that these pairs should not be held together during the whole year. When solitary species such as the riverine rabbits are given sufficient space they only exhibit close contact with conspecifics during the oestrous period or during the rearing of young (Eisenberg & Kleiman 1977). Although such species can coexist as pairs in captivity, when held in this fashion reproductive failure is common because the females may not conceive. Reproduction is successful only when the females are removed from proximity of the males except for the mating period

(Eisenberg & Kleiman 1977). It is therefore vital to obtain detailed information on the estrous season of riverine rabbits.

The intensive management of the captive breeding of the riverine rabbits is of vital importance. The rabbits need to be managed demographically to maintain a stable breeding population from which rabbits could be reintroduced into unoccupied but apparently suitable habitat. They need to be managed at the behavioural-ecological level to ensure that mating does indeed take place at an acceptable rate and they need to be managed genetically for ensuring the continued existance of the captive population as well as of the population of rabbits reintroduced into the wild.

Most importantly, the available natural habitat needs to be protected and the attitude of farm owners needs to be positive since these provide the essential framework within which successful reintroduction of rabbits into the wild can take place.

Genetic management is therefore only one aspect of a much larger and coordinated effort needed to prevent extinction of the riverine rabbit.

SUMMARY

The riverine rabbit (*Bunolagus monticularis*) occurs in the riparian scrub along the banks of seasonal rivers of the central Karoo. Over the last 40 to 50 years an estimated 60% of the original habitat has been destroyed to make way for agriculture. Estimates suggest that the remaining habitat could possibly support 1435 rabbits, but since rabbit densities vary widely along the same river, the actual total population size is probably much smaller (300 - 800). Therefore the riverine rabbit is endangered and in need of special conservation measures.

In an attempt to prevent the extinction of the riverine rabbit, a breeding programme was initiated at the De Wildt Cheetah Breeding Facility, with the aim of eventually releasing captive-bred rabbits back into the wild.

A breeding programme which would minimize the loss of heterozygosity was proposed (Appendix B). In order to determine which of the twelve rabbits in captivity would be suitable for inclusion as founders of the breeding programme, DNA fingerprinting was performed. This allowed the identification of those specimens that are the most genetically dissimilar from the remaining captive animals and which are presumably the least inbred. If fingerprint similarity reflects inbreeding, the least inbred rabbit would be 008.

An examination of semen revealed that only one male, specimen 002, produced sperm on both occasions of electroejaculation.

If a small number of rabbits must to be chosen for inclusion in the breeding programme the combined results of DNA fingerprinting and sperm counting suggest that specimens 001, 002, 008 and 014 should be selected. However, it is recommended that only a single rabbit from the captive population be used as a founder of the breeding programme since the other captive rabbits are relatively similar to the one chosen.

The similarity of fingerprints between the captive rabbits and a wild-captured rabbit from the same locality in the Karoo, suggested that subpopulations, or demes, of rabbits in the Karoo are also inbred. The speculated average heterozygosity of the population of riverine rabbits in the Karoo of approximately 0.02 was also lower than the average heterozygosity for mammals and is probably the result of the small expected effective population size, partly due to the subdivision of the Karoo population into at least two subpopulations. The small subpopulation west of the Fish River possibly has an average heterozygosity of approximately 0.034 which, together with its isolation from the rest of the population, makes it very susceptible to factors resulting in extinction. Therefore this subpopulation is in desperate need of genetic management. Because of the low heterozygosity and possible inbreeding within subpopulations it is suggested that the capture of founders for the breeding programme should be performed over a wide range of the geographic distribution of riverine rabbits.

OPSOMMING

Die rivierkonyn (*Bunolagus monticularis*) kom slegs in die rivierstruikgewasse op die oewers van die seisoenale riviere in die sentrale Karoo voor. Gedurende die afgelope 40 tot 50 jaar, is ongeveer 60% van die oorspronklike habitat, vir landboudoeleindes, vernietig. Na beraming, kan die oorblywende habitat waarskynlik slegs 1435 konyne huisves, maar aangesien die konyndigthede baie varieer langs dieselfde rivier, is die ware totale bevolkingsgrootte moontlik baie kleiner (300 - 800). Gevolglik is die rivierkonyn 'n bedreigde spesie en is spesiale bewaringsaksies noodsaaklik.

In 'n poging om die rivierkonyn van uitsterwing te red, is 'n teelprogram by die De Wildt Jagluiperd Teelstasie begin. Die uiteindelike doel hiermee is om konyne, wat in gevangenskap geteel is, in die natuur vry te laat.

'n Teelprogram wat die verlies van heterosigositeit sal minimaliseer, is voorgestel (Bylaag B). Deur middel van DNA vingerafdrukke is die verwantskappe van die twaalf konyne in gevangenskap bepaal om sodoende die indiwidue te identifiseer wat geskik sal wees as stigters van die teelprogram. Die keuse van stigters sal val op indiwidue wat geneties verskillend is van die ander en waar inteling laag is. Indien ooreenstemming in vingerafdrukke 'n weerspieëling van die mate van inteling is, is indiwidu 008 die konyn wat die minste ingeteel is.

'n Ondersoek van die semen het aan die lig gebring dat slegs een mannetjie (002), tydens beide elektro-ejakulasies, sperme

gelewer het.

Indien 'n klein aantal van die konyne gekies moet word vir insluiting by die teelprogram, dui die resultate van beide die DNA vingerafdrukke en die spermtelling op die volgende indiwidue: 001, 002, 008 en 014. Weens die groot genetiese ooreenkomste word egter aanbeveel dat slegs 'n enkele konyn, uit die bevolking in gevangenskap, as stigter gebruik word.

Die ooreenstemming tussen vingerafdrukke van die gevange konyne en 'n wilde konyn uit dieselfde lokaliteit in die Karoo, dui op die ingeteeldheid van subbevolkings in die Karoo. Die verdeling van die Karoo bevolking in minstens twee subbevolkings, het 'n verlaging van die effektiewe bevolkingsgrootte tot gevolg en dit het gelei tot 'n verlaging in die gemiddelde heterosigositeit. Die klein geïsoleerde subbevolking, wes van die Visrivier, het moontlik 'n heterosigositeitswaarde van 0.034 wat die bevolking baie kwesbaar maak. Die beraamde gemiddelde heterosigositeit van die bevolking rivierkonyne in die Karoo van ongeveer 0.02 is laer as die gemiddelde heterosigositeit van soogdiere. Daar word dus aanbeveel dat stigters vir die teelprogram oor 'n wye strook van hul geografiese verspreidingsgebied gevang moet word.

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APPENDIX A

```
{-----------------------------------------------[ PD_values ]----------}
PROGRAM PD_values;
{Die program lees molekulêre groottes van bande uit 'n ander lêer en    }
{bereken die "percentage difference" waardes tussen die individue.      }
{Die PD waardes word na 'n lêer geskryf, asook die totale bande en      }
{totale gelyke bande en nommers van die diere wat vergelyk is, asook die}
{bandgroottes en bandnommers van gelyke bande en die nommers van die 2  }
{diere wat vergelyk is. Die band naaste aan die 1 wat vergelyk word,    }
{word as die gelyke band gevat. Nie die eerste band binne mobiliteits=  }
{grens nie.                                                             }

{-----------------------------------------------[ Uses       ]----------}
  USES
    Crt
  {ENDUSES};

{-----------------------------------------------[ Const      ]----------}
  CONST
    maksdiere = 16;
    maksbande = 35
  {ENDCONST};

{-----------------------------------------------[ Type       ]----------}
  TYPE
    gt_bandindeks    = 0..maksbande;
    gt_dierindeks    = 0..maksdiere;
    gt_naamskik      = ARRAY [gt_dierindeks] OF INTEGER;
    gt_tot_bandskik  = ARRAY [gt_dierindeks] OF gt_bandindeks;
    gt_bandmatr      = ARRAY [gt_bandindeks, gt_dierindeks] OF REAL;
    gt_PDmatr        = ARRAY [gt_dierindeks, gt_dierindeks] OF REAL
  {ENDTYPE};

{-----------------------------------------------[ Var        ]----------}
  VAR
    gv_bandskik  :  gt_tot_bandskik;
    gv_bandmatr  :  gt_bandmatr;
    gv_PDmatr    :  gt_PDmatr;
    gv_naamskik  :  gt_naamskik;
    InLeerNaam   :  STRING[40];
    UitLeerNaam  :  STRING[40];
    StoorLeer    :  STRING[40];
    DataLeer     :  STRING[40];
    antwoord     :  char
  {ENDVAR};

{-----------------------------------------------[ Inis  P1   ]----------}
  PROCEDURE Inisialiseer (VAR up_bandskik   :  gt_tot_bandskik;
                          VAR up_bandmatr   :  gt_bandmatr;
                          VAR up_PDmatr     :  gt_PDmatr;
                          VAR up_naamskik   :  gt_naamskik);
  {Hierdie prosedure inisialiseer die matrikse en skikkings wat gebruik }
  {gaan word en stel al die waardes gelyk aan 0. 2 Skikkings nl. vir     }
  {die individue se nommers en totale aantal bande en 2 matrikse nl. vir}
  {die bandgroottes en die berekende PD-waardes word gebruik.            }

    {-----------------------------------------------[ Var        ]----------}
    VAR
      diernr    :  gt_dierindeks;
```

```
    teldier  :  gt_dierindeks;
    bandnr   :  gt_bandindeks
  {ENDVAR};

  BEGIN
    {--------------Inisialiseer beide skikkings-----------------------}
    FOR diernr := 1 TO maksdiere DO
      BEGIN
        up_naamskik[diernr] := 0;
        up_bandskik[diernr] := 0;
      END
    {ENDFOR};
    {--------------Inisialiseer PD matriks----------------------------}
    FOR diernr := 1 TO maksdiere DO
      BEGIN
        FOR teldier := 1 TO maksdiere DO
          BEGIN
            up_PDmatr[teldier, diernr] := 0;
          END
        {ENDFOR};
      END
    {ENDFOR};
    {--------------Inisialiseer bandgrootte matriks-------------------}
    FOR bandnr := 1 TO maksbande DO
      BEGIN
        FOR diernr := 1 TO maksdiere DO
          BEGIN
            up_bandmatr[bandnr, diernr] := 0;
          END
        {ENDFOR};
      END
    {ENDFOR};
  END
{END Inisialiseer};

{-----------------------------------------------[ LeesInvoer P2 ]------}
PROCEDURE LeesInvoer (VAR iup_bandskik   :  gt_tot_bandskik;
                      VAR iup_naamskik   :  gt_naamskik;
                      VAR iup_bandmatr   :  gt_bandmatr);
{Alle data word uit 'n teks lêer uit in die verskillende skikkings en }
{in die bandgrootte matriks ingelees.                                  }

  {-----------------------------------------[ Var       ]----------}
  VAR
    diere_leer  :  TEXT;
    diernr      :  gt_dierindeks;
    bandnr      :  gt_bandindeks
  {ENDVAR};

  BEGIN
    {-------------------------Open lêer-------------------------------}
    ASSIGN (diere_leer, 'A:'+InLeerNaam);
    RESET (diere_leer);
    {-------------------Lees ind se nr en totale aantal bande---------}
    FOR diernr := 1 TO maksdiere DO
      BEGIN
        READLN (diere_leer, iup_naamskik[diernr], iup_bandskik[diernr]);
        FOR bandnr := 1 TO iup_bandskik[diernr] DO
          BEGIN
            {----------Lees bandgroottes ----------------------------}
```

```
            READLN (diere_leer, iup_bandmatr[bandnr, diernr]);
          END
        {ENDFOR};
      END
    {ENDFOR};
    {-------------------------Sluit lêer------------------------------}
    CLOSE (diere_leer);
  END
{END LeesInvoer};

{-----------------------------------------[ Verwerk P3 ]---------}
PROCEDURE Verwerk (ip_bandskik    : gt_tot_bandskik;
                   ip_naamskik    : gt_naamskik;
                   ip_bandmatr    : gt_bandmatr;
                   VAR iup_PDmatr : gt_PDmatr);
{Hierdie prosedure vergelyk al die bande van al die individue met      }
{mekaar en bereken die PD-waardes en skryf dit in die PD-matriks in.   }
{Die ander 2 lêers word ook in hierdie prosedure geskryf               }

  {-----------------------------------------[ Var       ]----------}
  VAR
    diernr, teldier              :  gt_dierindeks;
    bandnr, telband, matchband   :  gt_bandindeks;
    somgelyk                     :  BYTE;
    somband                      :  BYTE;
    somverskil                   :  BYTE;
    PD, D                        :  REAL;
    PDsom, APD                   :  REAL;
    hoevPD                       :  INTEGER;
    x                            :  1..10;
    match_leer, ind_leer         :  TEXT;
    kleinste, bandverskil        :  REAL
  {ENDVAR};

  BEGIN
    WRITELN;
    {--------------Die % mobiliteitsverskil moet ingelees word--------}
    WRITELN ('Sleutel die mobiliteitsverskil, wat in ag geneem moet word, in: ');
    WRITELN ('Mobiliteitsverskil van 1% tot 10% ');
    WRITELN ('Net die getal en dan ENTER.');
    READLN (x);
    WRITELN;
    WRITELN ('Wees geduldig !!! Ek WERK !!! ');
    {--------------Inisialiseer alle veranderlikes--------------------}
    teldier := 0;
    telband := 1;
    diernr  := 1;
    bandnr  := 1;
    somgelyk := 0;
    somband := 0;
    somverskil := 0;
    D := 0;
    PD := 0;
    hoevPD := 0;
    PDsom := 0;
    APD := 0;
    {-------------------Open lêers-----------------------------------}
    ASSIGN (match_leer,'a:'+DataLeer);
    REWRITE(match_leer);
    ASSIGN (ind_leer,'a:'+StoorLeer);
```

```
REWRITE(ind_leer);
{------Vergelyk alle indiwidue met mekaar-------------------------}
FOR diernr := 1 TO (maksdiere - 1) DO
  BEGIN
    {-----Vergelyk 1 ind met alle volgende indiwidue--------------}
    FOR teldier := (diernr + 1) TO maksdiere DO
      BEGIN
        {-----Vergelyk alle bande van 1 ind met vlg ind-----------}
        matchband := 0;
        FOR bandnr := 1 TO ip_bandskik[diernr] DO
          BEGIN
            {-----Vergelyk 1 band met alle bande in vlg ind-------}
            {------net vanaf band na die vorige gelyke band-------}
            FOR telband := matchband + 1 TO ip_bandskik[teldier] DO
              BEGIN
                {-----Bring mobiliteitsverskil in aanmerking------}
                IF (ip_bandmatr[telband, teldier] <= (ip_bandmatr[bandnr, diernr]
                   + (ip_bandmatr[bandnr, diernr] * (x/100)))) AND
                   (ip_bandmatr[telband, teldier] >= (ip_bandmatr[bandnr, diernr]
                   - (ip_bandmatr[bandnr, diernr] * (x/100))))
                  THEN
                    BEGIN
                      matchband := telband;  {-----merk gelyke band----}
                      kleinste := 100;
                      bandverskil := ABS(ip_bandmatr[bandnr, diernr] - ip_bandmatr[telband,
                                     teldier]);
                      {-----------Toets vir naaste band-----------}
                      WHILE (bandverskil < kleinste) AND ((ip_bandmatr[telband, teldier]
                            <= (ip_bandmatr[bandnr, diernr] + (ip_bandmatr[bandnr, diernr]
                            * (x/100)))) AND (ip_bandmatr[telband, teldier] >=
                            (ip_bandmatr[bandnr, diernr]
                            - (ip_bandmatr[bandnr, diernr] * (x/100))))) DO
                         BEGIN
                           kleinste := bandverskil;
                           matchband := telband;
                           telband := telband + 1;
                           bandverskil := ABS(ip_bandmatr[bandnr, diernr] -
                                          ip_bandmatr[telband, teldier]);
                         END
                      {ENDWHILE};
                      {-----Skryf diernrs wat vergelyk word en bande wat gelyk is---}
                      WRITE(match_leer, diernr:4,' ',teldier:4);
                      WRITELN(match_leer,ip_bandmatr[bandnr, diernr]:8:4,' ',
                              ip_bandmatr[matchband, teldier]:8:4,' ',
                              bandnr,' ',matchband);
                      somgelyk := somgelyk + 1;  {---Tel gelyke bande---}
                      telband := ip_bandskik[teldier]; {---Einde laan---}
                    END
                  ELSE
                    {---As bande wat vergelyk moet word <, gaan na einde van bande-}
                    IF (ip_bandmatr[telband, teldier] < (ip_bandmatr[bandnr, diernr]
                       - (ip_bandmatr[bandnr, diernr] * (x/100))))
                      THEN
                        telband := ip_bandskik[teldier] {----Einde laan----}
                    {ENDIF}
                {ENDIF};
              END
            {ENDFOR};
          END
        {ENDFOR};
```

```
            {--Bereken totale aantal bande van 2 ind wat vergelyk word-}
            {-------Skryf na lêers-------------------------------------}
            WRITELN(ind_leer, ip_naamskik[diernr]:5, ip_naamskik[teldier]:5,
                    ip_bandskik[diernr]:5, ip_bandskik[teldier]:5, somgelyk:5);
            somband := ip_bandskik[diernr] + ip_bandskik[teldier];
            {--Bereken aantal bande wat verskil tussen 2 ind----------}
            somverskil := somband - somgelyk * 2;
            IF somband <> 0
              THEN
                BEGIN
                  {-----Bereken verskil tussen 2 ind------------------}
                  D := somverskil / somband;
                  {-----Bereken persentasie verskil-------------------}
                  PD := D * 100;
                  hoevPD := hoevPD + 1;
                  PDsom := PDsom + PD;
                  {-----Skryf na matriks------------------------------}
                  iup_PDmatr[diernr, teldier] := PD;
                  somgelyk := 0;
                END
              ELSE
                WRITELN('Deling deur 0.');
          END
        {ENDFOR};
      END
    {ENDFOR};
    APD := PDsom/hoevPD;
    WRITELN ('Die APD value is: ',APD:5:2);
    CLOSE (match_leer);
    CLOSE (ind_leer);
  END
{END Verwerk};

{-----------------------------------------------[ Skryfleer P4 ]-------}
PROCEDURE Skryfleer (VAR iup_PDmatr   : gt_PDmatr;
                     VAR iup_naamskik : gt_naamskik);
{Hierdie prosedure skryf die indiwidue se nommers en PD-waardes na     }
{'n tekslêer.                                                          }

  {-------------------------------------------[ Var        ]----------}
  VAR
    PD_leer  :  TEXT;
    diernr   :  gt_dierindeks;
    teldier  :  gt_dierindeks;
    pdval    :  REAL
  {ENDVAR};

  BEGIN
    diernr := 1;
    {---------------Open lêer-------------------------------------------}
    ASSIGN (PD_leer, 'A:'+UitLeerNaam);
    REWRITE (PD_leer);
    {---------------Skryf ind se nommers-------------------------------}
    FOR diernr := 1 TO maksdiere DO
      BEGIN
        WRITELN (PD_leer, iup_naamskik[diernr]:4);
      END
    {ENDFOR};
    WRITELN(PD_leer);
    diernr := 1;
```

```
    {-------------Skryf ind se PD-waardes-----------------------------}
    FOR diernr := 1 TO maksdiere DO
      BEGIN
        FOR teldier := 2 TO maksdiere DO
          BEGIN
            pdval := iup_PDmatr[diernr, teldier];
            IF pdval = 0
              THEN
                WRITE(PD_leer,'      ')
              ELSE
                WRITE (PD_leer, pdval/100:5:3,' ')
            {ENDIF};
          END
        {ENDFOR};
        WRITELN(PD_leer);
      END
    {ENDFOR};
    {-----------------Sluit leer-------------------------------------}
    CLOSE (PD_leer);
  END
{END Skryfleer};

{=====================================================================}
{                          M  A  I  N                                 }
{=====================================================================}

BEGIN
  Inisialiseer (gv_bandskik, gv_bandmatr, gv_PDmatr, gv_naamskik);   {P1}
  Crt.CLRSCR;
  WRITELN('SUSAN SE SUPER-DUPER DNA VINGERAFDRUK-PROGRAM');
  WRITELN('-----------------------------------------');
  WRITELN;
  REPEAT
    WRITE('(S)nap / (P)ucj ? ');
    antwoord := ReadKey;
    CASE UpCase(antwoord) OF
        'S': BEGIN
               InLeerNaam := 'SNAP.DAT';
               UitLeerNaam := 'PDSNAP.DAT';
               StoorLeer := 'INDSNAP.DAT';
               DataLeer := 'MATSNAP.DAT';
               WRITELN;
               WRITELN('Invoer uit SNAP.DAT');
               WRITELN('Gelyke bande na MATSNAP.DAT');
               WRITELN('Uitvoer na PDSNAP.DAT');
               WRITELN('Inligting omtrent indiwidue na INDSNAP.DAT');
             END;
        'P': BEGIN
               InLeerNaam := 'PUCJ.DAT';
               UitLeerNaam := 'PDPUCJ.DAT';
               StoorLeer := 'INDPUCJ.DAT';
               DataLeer := 'MATPUCJ.DAT';
               WRITELN;
               WRITELN('Invoer uit PUCJ.DAT');
               WRITELN('Gelyke bande na MATPUCJ.DAT');
               WRITELN('Uitvoer na PDPUCJ.DAT');
               WRITELN('Inligting omtrent indiwidue na INDPUCJ.DAT');
             END;
        ELSE
           BEGIN
```

```
                WRITELN;
                WRITELN('KIES ASSEBLIEF ''S'' OF ''P''');
                WRITELN;
              END;
          END
        {ENDCASE};
        UNTIL (UpCase(antwoord) = 'S') OR (UpCase(antwoord) = 'P');
      WRITELN;
      WRITELN ('Data word uit invoerlêr gelees.');
      LeesInvoer (gv_bandskik, gv_naamskik, gv_bandmatr);                    {P2}
      WRITELN;
      WRITELN ('Data word verwerk.');
      Verwerk (gv_bandskik, gv_naamskik, gv_bandmatr, gv_PDmatr);            {P3}
      Skryfleer (gv_PDmatr, gv_naamskik);                                    {P4}
      WRITELN;
      WRITELN ('VERWERKING VOLTOOI !!!!!!!!! ');
      DELAY (2000);
    END
{END PDvalues}.
{=============================================================================}
```